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C. F. A. PANTIN, Sc.D., F.R.S.
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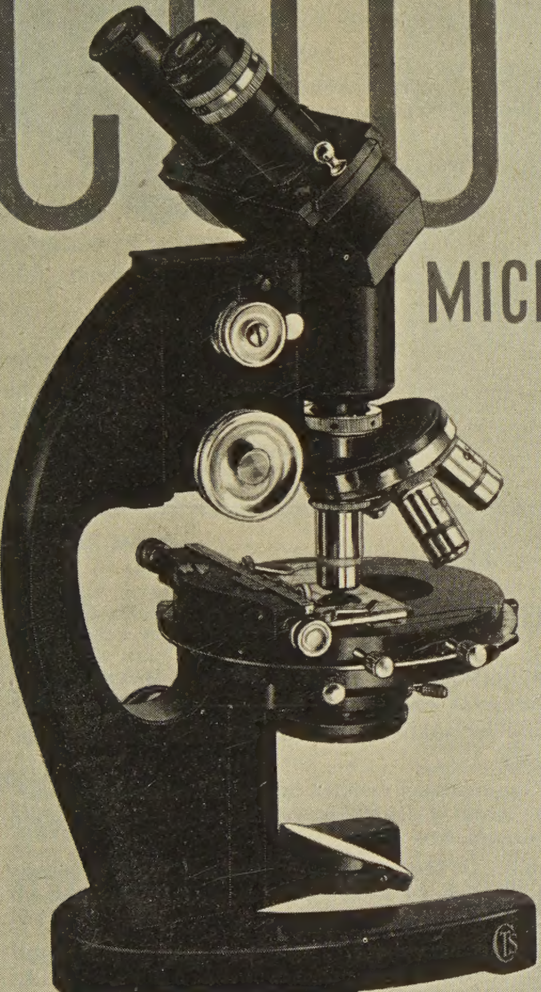

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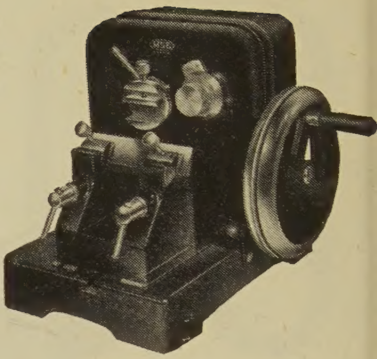

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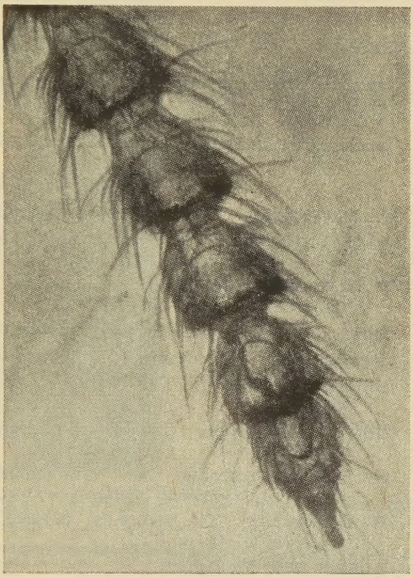

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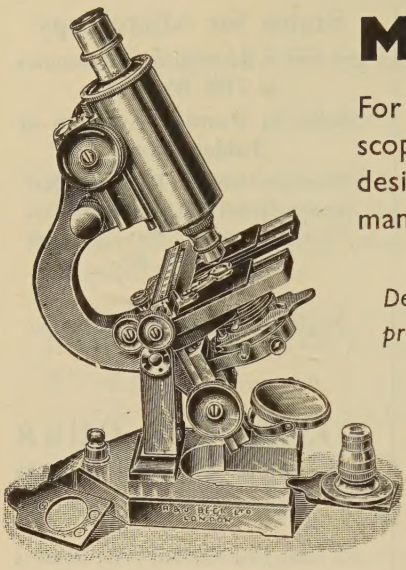

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The Developmental Cycle of Botrylloides

BY

N. J. BERRILL
(*McGill University, Montreal*)

With five Text-figures

OF the many studies made on ascidians in the past, few have included the organism in its entirety. The present account is of the whole developmental cycle of an organism exhibiting exquisite timing in its four-dimensional relationships, with an interpretation of a colonial organism as the result of a few interacting processes. A comparison of *Botrylloides* with *Botryllus* affords some analysis of the nature of differences of generic magnitude, as modifications of several basic activities.

Botrylloides is an ascidian so clearly related to *Botryllus* that it has often been relegated to the status of a subgenus of that form. Only a few species are known and species differences are very small indeed. The type discussed here is *Botrylloides leachi* Savigny; others are *B. diegense* of the North American Pacific coast, stated by Van Name (1945, p. 230) to be an extremely close ally of *B. leachi*, *B. aureum*, a more northern form differing mainly in the position of the sperm duct opening and in colour range, and *B. nigrum* of the West Indies that personal investigation has shown to be essentially the same as *B. leachi* except for colour range. Species of the genus *Botryllus* are almost equally restricted in number and order of differences, except that in two species the number of rows of stigmata is reduced to four. We can accordingly speak in the more general terms of reference to the genus *Botrylloides* and the genus *Botryllus* and to the nature and origin of the generic differences between them. Most of the peculiarities of *Botrylloides* represent specializations of features present in *Botryllus*, and with some reserve the assumption is made that there has been an actual evolution from *Botryllus* itself, the latter showing little evidence of independent specializations of its own, and there seems to be no need to introduce a hypothetical common ancestor significantly different from it.

STRUCTURE OF ZOOID

The zooid structure is in general similar to that of *Botryllus* zooids and, apart from the nature of the atrial siphon, very much like zooids of *Symplegma* (Berrill, 1940). In all three the branchial sac is secondarily simple, the four folds typical of the Polystylidae being reduced to three, and each fold represented by a single internal longitudinal vessel. Likewise the gonads, characteristically scattered as polycarps over the atrial wall, are present as a single hermaphrodite unit on each side. The branchial sac opens into the looped digestive tube, of which the stomach possesses longitudinal hepatic folds. The heart is compact and lies on the opposite side close to the base of the endostyle. The peribranchial sacs, diverging from the atrial chamber along the left and right sides of the branchial sac, extend beyond the abdomen to meet posteriorly, though without fusing. In consequence, all the visceral organs are separated from circulating sea-water by a simple epithelial membrane.

The distinctive features of the *Botrylloides* zooid are the more elongate shape of the branchial sac, due to relatively more rows of stigmata and fewer stigmata per row, the wide atrial siphon, the orientation of the gonad, the ovary being ventral instead of lateral to the testes, and the placental connexion with the solitary developing embryo on each side (fig. 1 B, C).

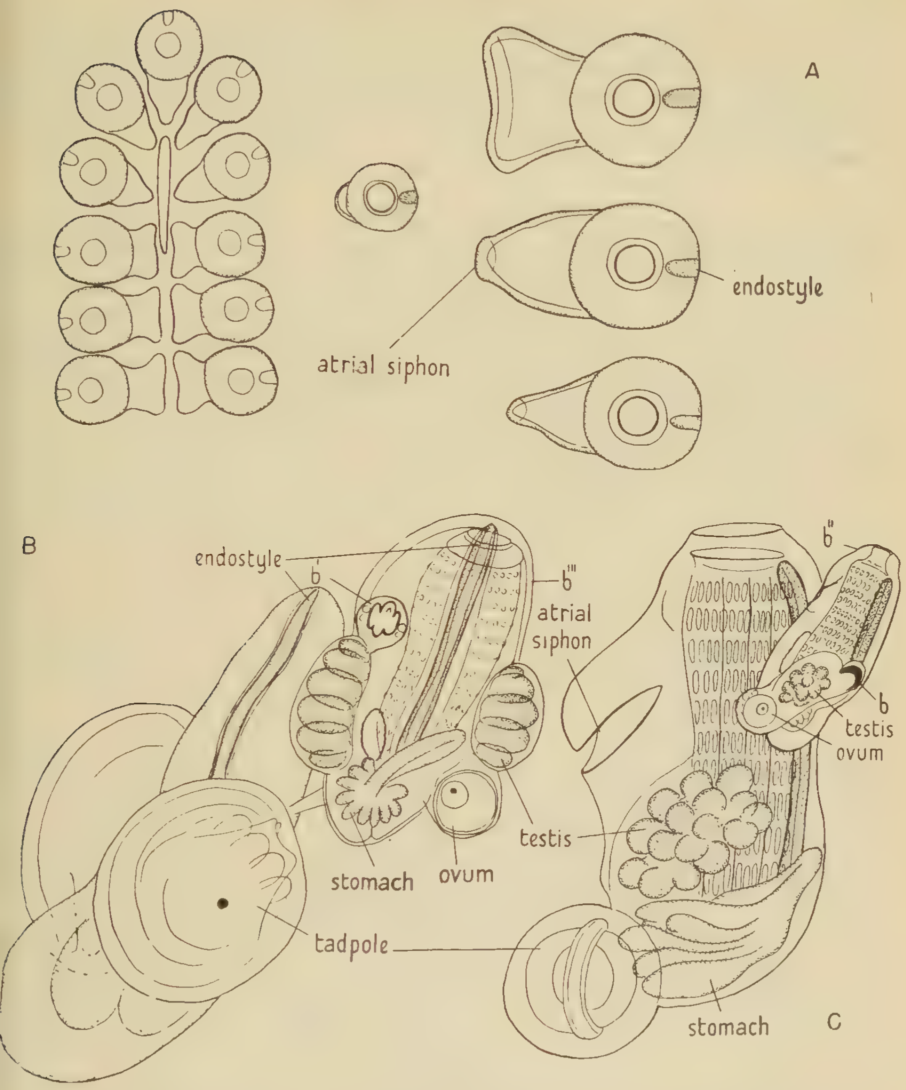
ATRIAL SIPHON AND THE FORMATION OF SYSTEMS

In *Symplegma* atrial siphons are not hypertrophied relative to the branchial siphons, and no systems are formed. In *Botryllus* the atrial siphons are enlarged and conical, and buds developing adjacently to one another orientate in the final stages to form typical star-shaped systems, the narrow ends of the atrial cones opening into the common cloacal chamber of each system. In *Botrylloides* the atrial siphon exhibits a greater range of form. In very small individuals, of the first few asexual generations, the atrial siphons are no larger than the branchial, and no systems are formed. When somewhat larger, conical siphons develop and there is a tendency to produce star-systems as in *Botryllus*. In mature *Botrylloides* colonies, however, two types coexist, square-edged forms that can inevitably form only ladder-like systems, and conical forms that comprise the ends of the separate ladder systems. The two together account both for the existence of the ladder system and its limits, while the progressive change in size and form of the atrial siphon seems to be a typical case of relative growth (fig. 1 A).

BUDDING CYCLE

Buds are formed in *Botrylloides* at the same place and stage as in *Botryllus*. At the stage when stigmata are visible merely as imperforate rows, a bud disk appears on each side mainly involving the atrial epithelium. When the parent bud is almost half-grown, its buds already exhibit the principal morphological divisions, namely, the branchial, peribranchial, neural, and ovarian units. When fully grown and just functioning, a zooid bears buds that are a little younger than the stage at which the subsequent bud generation appears. In the final phase of bud growth, which is primarily one of cell growth and differentiation, in contrast to cell proliferation, the bud draws so heavily on the nutritional supply from its parent that the latter undergoes resorption (fig. 1 B).

There is accordingly a very definite cycle of bud initiation, development, and growth, followed by a period of survival as a functional organism, ending when the buds of the next generation attain in turn their functional maturity.



TEXT-FIG. 1.

A. Upper surface views of zooids isolated and arranged in a system, showing correlation between size and shape of the atrial siphon and relation to pattern of system, the central figure representing the condition in the first blastozooid generation. B, C. Two zooids from mature colonies showing coexistence and stages of three bud generations in each case. In B the functional zooid is atrophying and about to liberate fully developed tadpoles by rupture, its bud (*b‴*) has perforating stigmata, has a testis on its left side and testis plus ovary on the right, and carries a bud (*b′*) of the next generation on its right side (cp. text-fig. 2C). C shows functional zooid with testis and young tadpole within brood pouch, bud (*b″*) of next generation on its left side with non-perforate stigmata and testis and ovary, together with its own bud (*b*) in late disk stage.

The whole cycle is constant in time for any given temperature, and every stage in the cycle is correlated with an equally well-defined stage of the preceding and, in later stages, of the succeeding generation. As in *Botryllus*, small buds and large buds develop at approximately the same rate and endure as functional zooids for the same period, this last being 8 or 9 days at a temperature of 16°–17° C.

Bud Development

Buds arise from the atrial wall on either side, about midway along the antero-posterior axis and somewhat toward the ventral side. They appear at a precise stage in the development of the parent zooid, when stigmata rows are represented by imperforate bands of tissue, and arise first as disks of thickened atrial epithelium immediately anterior to the testes. When first discernible the bud disk is three or four cells in diameter, but in the case of those destined to become sexually mature zooids, the disk grows as such until there are about twelve cells in optical section. Then the disk, together with a corresponding area of overlying epidermis, arches progressively until the two layers form an inner closed sphere of atrial epithelial cells and an outer epidermal sphere connected with the parent epidermis by a narrow stalk. At this simplest of morphological stages, two features suggest that the simplicity of pattern is more apparent than real, for an ovum, if one is to be produced at all, is already segregated from the wall of each side of the inner vesicle, while the distal region of the epidermal vesicle shows a slight bulge foreshadowing the ampulla which eventually attaches the bud to the colonial vascular system. This last feature may, of course, be merely a response to the local impinging of the blood-stream circulating between the two vesicles, inducing a local epidermal outgrowth (fig. 2 A, B).

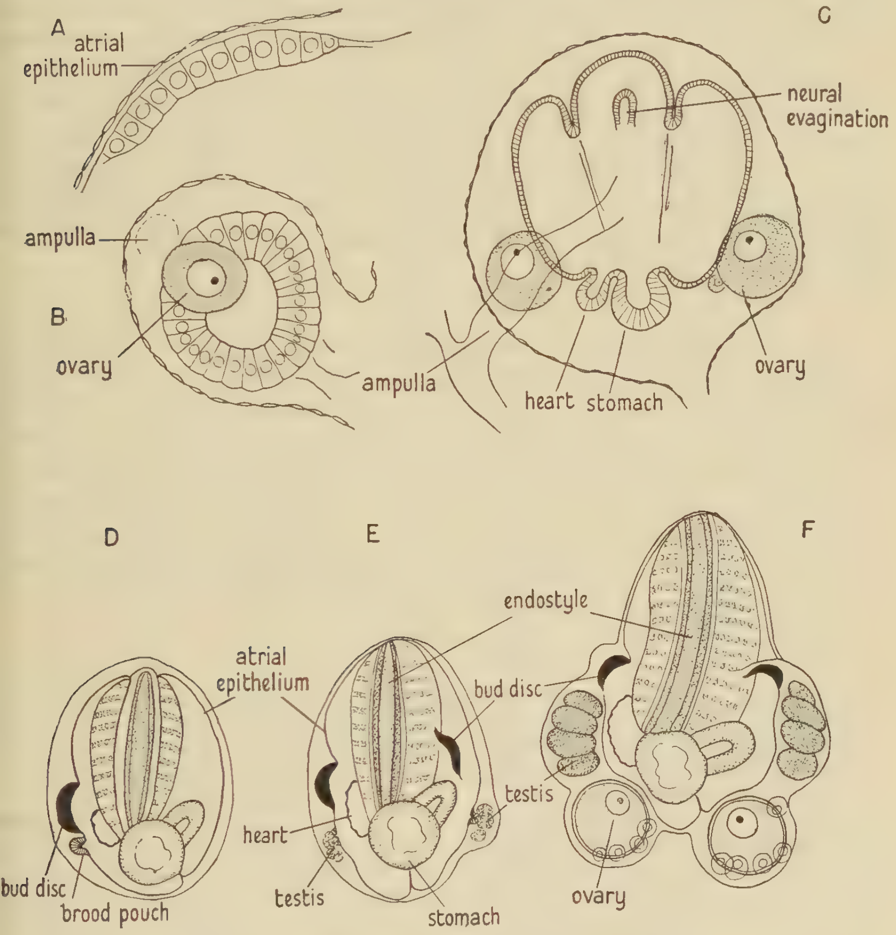
As the bud grows in size, the inner vesicle, when two to three times its original diameter, exhibits all the primary morphological divisions in the form of infoldings and outpushings. A pair of lateral folds, commencing anteriorly, divide the primary vesicle into a central pharyngeal chamber and two lateral atrial chambers. Dorsally a median evagination forms the neural complex, while posteriorly two evaginations form the rudiments of the heart and the digestive tube. At the same time the epidermal ampulla foreshadowed earlier has grown and fused with the nearest adjacent vessel of the colonial vascular system (fig. 2 C).

This stage grows without marked change other than differentiation of the established units until it is several times as large. The heart becomes cut off as a closed vesicle, the intestinal evagination lengthens to form the gut loop, and the midventral band of the pharynx differentiates as endostyle.

The next critical stage is that in which the rows of stigmata are indicated by imperforate transverse bands and the buds of the next generation appear as atrial disks. The new buds follow the course just described, the parent bud continuing to grow until several times as large, to attain functional differentiation with little further morphological change (fig. 2 E).

INITIAL BUD SIZE AND DEVELOPMENT

The relationship between the initial size of buds as represented by the maximum disk stage and the size of subsequent stages including the final one



TEXT-FIG. 2. Development of bud.

. Bud disk at maximum stage showing epidermal and atrial layers. B. Bud sphere with ovum already segregated from wall of inner layer. C. Young bud with ova segregated on each side, ampullary outgrowth attached to colonial vascular system, and inner vesicle folding to form pharyngeal and atrial divisions and neural, cardiac, and intestinal outgrowths. D, E, and F. Three later buds of different size but identical stage showing correlation of absolute size with presence of one or two buds, and variation in gonads.

s direct, and similar to that reported for *Botryllus* (Berrill, 1940). Three izes of equivalent stages from a single colony and belonging to the same bud eneration are shown. All possess maximal bud disks of the new generation, nd stigmata are recognizable but not perforate. The bud developing from he smallest disk has its own bud disk only on its right side and has no trace of gonads. In the case of the disk of intermediate size, the bud has developed atrial disks and also rudimentary testes on each side, but no ovaries (fig. 2 D, E).

The largest disk has produced a maximal bud of the same stage, with bud disk, testes, and ovary on each side. The testes are destined to become functional and consist of several follicles. The ovary consists of one large ovum, and several rudimentary ova which do not grow and eventually degenerate and disappear (fig. 2 F).

These differences are correlated with the size of the bud during the transformation from the disk to the closed vesicle stage, when the large ova and, immediately following, the parental testis and small ova cells are segregated from the vesicle wall. Limitations of size below certain values prohibit such segregation and lead to the results exhibited in the small-disk buds just described.

DOUBLE BUDS

Double buds frequently are formed during the development of maximal buds in well-nourished colonies, apparently on the right side only. They arise from the division of a single rudiment during the late disk stage, one member of the pair forming a maximal bud (and zooid) similar to the bud on the left side, the other always smaller and rarely producing ova or functional testes. The inequality comes from the division of a rudiment of somewhat pear-shaped area, a large and small circular bud area deriving from the large and small ends respectively. All buds, large or small, of the same generation, complete their development and function simultaneously.

GONADS AND PLACENTATION

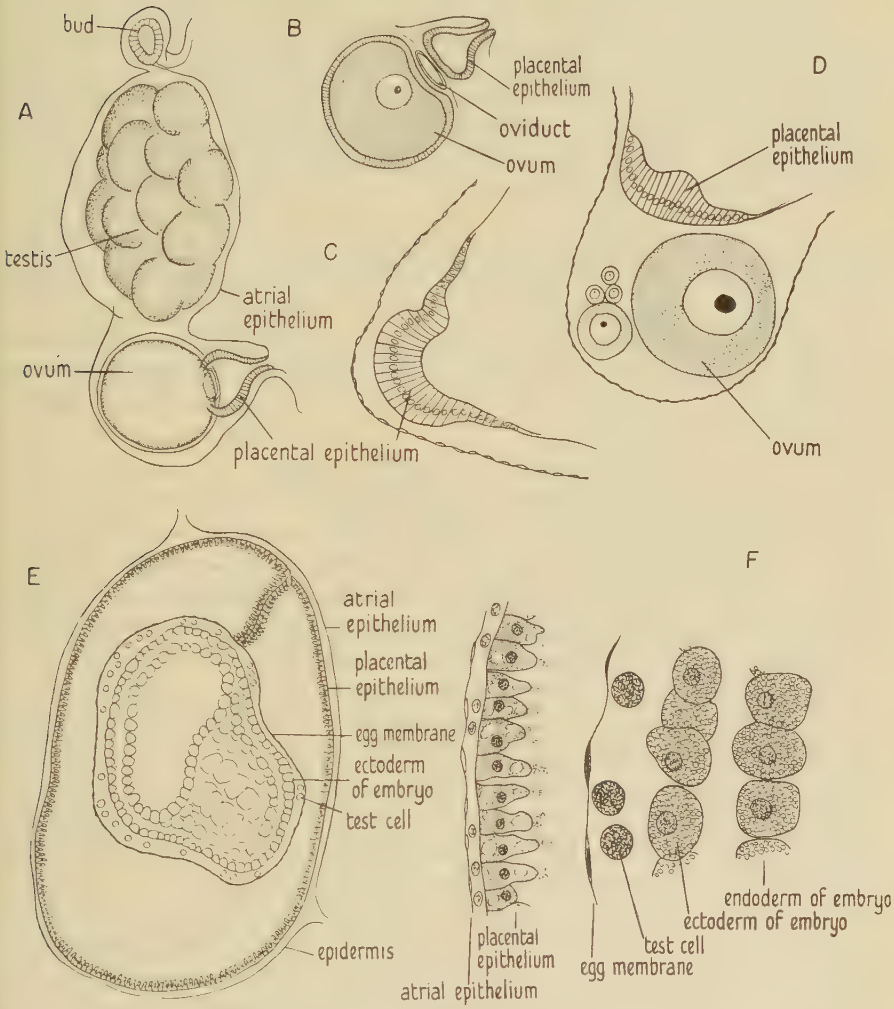
There has been considerable discussion concerning both the nature of the ovary and of the placenta, different descriptions and interpretations having been presented by Ärnbäck (1923) and by Garstang and Garstang (1928).

According to Ärnbäck,

> 'a brood pouch develops on each side of the body to receive the embryos. The brood-pouch is of globular form and is a diverticulum of the peribranchial cavity. The oviduct opens into the brood pouch. The embryos undergo their larval development in these uterus-like pouches, in which also fertilization takes place. The egg passes through the oviduct into the brood pouch; before entering the latter the outer follicle has been thrown off, forming a corpus luteum-like structure that lies in the mesoderm and soon degenerates.'

Garstang and Garstang describe the same thing very differently.

> 'When the egg is ready for fertilization the outer follicle grows out towards the atrial epithelium as a short thick tube. The duct is probably the homologue of the oviduct of other ascidians, but here it functions simply as a fertilization duct. The eggs are not shed into the atrium. Development takes place *in situ*, that is, the larvae are found between ectodermal and atrial walls, in a blood space. . . . The larvae are surrounded by the inner and outer follicles. . . . When a larva is almost ready for emergence, both follicles and the atrial epithelium lying close against them are



TEXT-FIG. 3. Placental brood pouch.

A. Reproductive complex with anterior bud (at sphere stage), median testis, and posterior ovary consisting of single ripe ovum about to enter brood pouch. B. Developing ovum with germinal vesicle and follicle layer, reduced oviduct, and brood pouch. C. Early stage in development of brood pouch from young bud without sign of gonad. D. Similar stage of brood pouch adjacent to young oocytes. E. Section through functional brood pouch and contained embryo. F. Higher magnification of layers of brood pouch and embryo.

broken through and the larva slips into the atrium. Botrylloides has remarkably little yolk in its eggs, but throughout the embryonic stages, up to the time when the larva frees itself from the follicle, a small tube is invariably present, which connects the outer with the inner follicle. This tube is formed of cubical cells, with clear rounded ends projecting towards the lumen—very like the cells of the outer follicle. At its outer end the tube opens into the bloodspace surrounding the larva, and as this is continuous with the visceral blood-space of the parent, something like a placental communication is established, though the exact way in which nourishment is transmitted is obscure. In Botryllus there is no such tube, and no need of it, because the eggs are provided with a good deal of yolk.'

Commenting on Ärnbäck's account, they suggest that the Plymouth variety of *Botrylloides* is more specialized for vascular nutrition than the Swedish variety described by Ärnbäck.

Both interpretations suffer from being based on insufficient material. All structures described by Ärnbäck and Garstang and Garstang have been identified in Plymouth material, but the account given below confirms and goes beyond that of Ärnbäck.

Shortly before the egg matures it is seen to possess both inner and outer follicle layers, with the germinal vesicle on the side nearest the atrium, and a short oviduct adjacent to that side. This condition is very similar to that of *Botryllus* at the same stage. In *Botryllus*, somewhat later, the egg emerges through the oviduct into the atrium but remains attached by a cup-shaped fold of the atrial wall at the site of emergence, at least until the completion of gastrulation. In *Botrylloides* a flask-shaped invagination of thickened atrial epithelium comes into contact with the oviduct and ovum before maturation, with the passage to the atrium narrowly constricted. This is in conformity with Ärnbäck's description (fig. 3 A, B).

Subsequently the ovum, immediately following the attainment of functional activity by the developing zooid as a whole, ruptures the outer follicle, enters the oviduct and then the atrial involution. This also confirms Ärnbäck, although the discarded outer follicle has not been seen.

These events occupy a very short while, probably not more than 2 or 3 hours. This is a relatively brief part of the whole cycle, and Garstang and Garstang apparently missed the critical period. All the zooids in any one colony are at the same stage and progress in unison through the cycle.

What actually occurs is that the ovum, upon rupturing the follicle, matures and becomes fertilized as it reaches the atrial brood pouch. It retains its inner follicle with basal chorion, together with the so-called test cells in the perivitelline space. Fluid accumulates between the embryo (with its membrane or chorion) and the lining of the brood pouch so that the pouch becomes distended. As it swells it protrudes both out from the body-wall of the zooid and into the atrial cavity, and in so doing the neck of the pouch which originally communicated with the atrial cavity becomes compressed, with its lumen obliterated and the atrial connexion lost. The inner end of the neck remains close to the egg membrane, so that it becomes virtually invaginated in the expanding pouch. It has almost but not entirely the appearance of a duct and is the placental tube of Garstang and Garstang. Accordingly the structure is a relic of no further functional significance and there is left the problem of the origin and nature of the pouch proper (fig. 3 E).

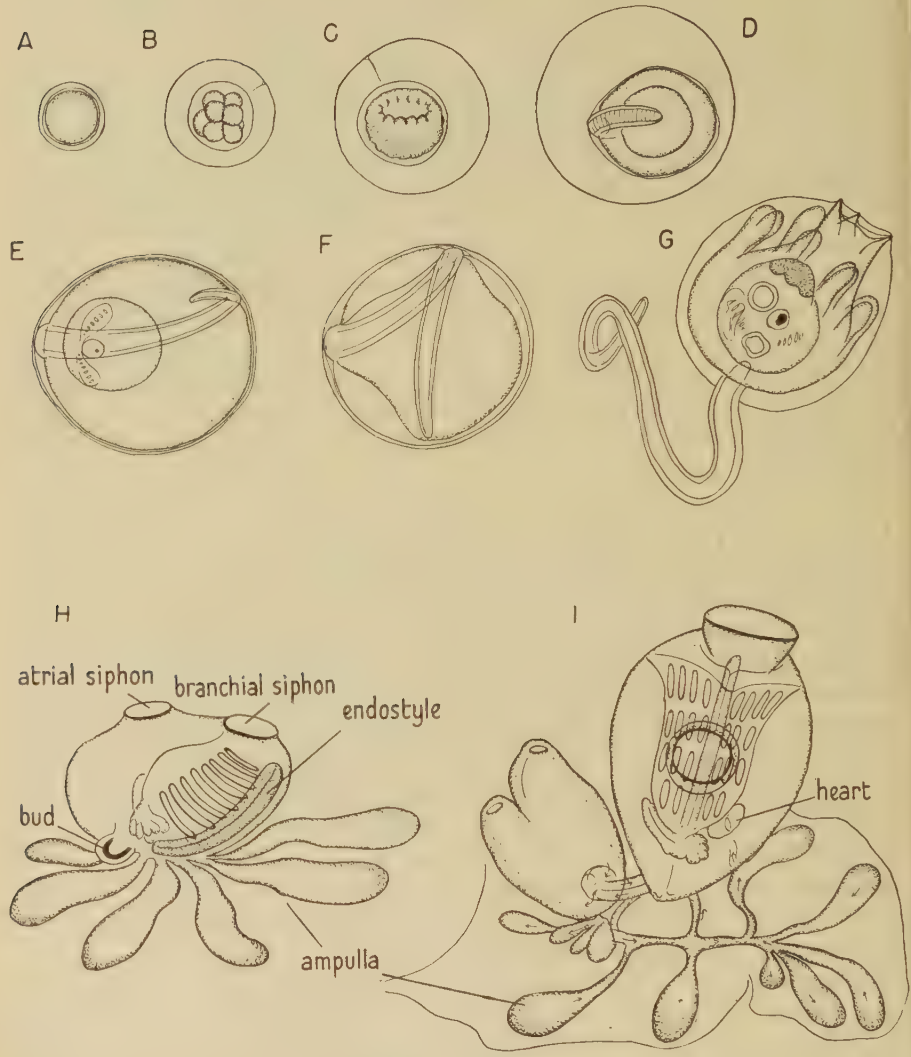
The pouch arises in the beginning as a thickening of atrial epithelium during the early development of the bud, at about the same time as the appearance of the stigmata anlagen and the bud disks of the next generation. The thickening, which represents local growth, appears adjacent to the ovary, but as far as can be determined it develops at least to some degree even when no ovary is present, and accordingly is not a dependent formation (fig. 3 C, D).

The columnar nature of the epithelium is retained throughout its existence into the functional state. In a brood pouch containing a developing embryo the lining cells are high columnar, vacuolated, rich in secretory granules, and appear to have plasmodial ends. They clearly secrete a coaguable substance into the interior of the pouch. Throughout the period of development of the egg the fluid increases and the pouch becomes more and more distended (fig. 3 E, F).

EMBRYONIC DEVELOPMENT

Basically the development of the egg of *Botrylloides* is like that of *Botryllus* up to and including the tadpole stage, with two obvious differences. These are the relatively small size of the *Botrylloides* egg, and the relatively large and precociously formed space formed within the archenteron and between the endoderm and ectoderm. These two features appear to be associated with the extra-embryonic nutrition, the pressure of the external fluid invading the embryo and in a sense producing an oedema between the layers, and the external nutrition compensating for reduction in egg size to produce the same end result. Actual nourishment of the developing embryo by the secretory lining of the brood pouch is, of course, not proven, and further investigation along physiological lines is desirable. There is, however, an active secretion towards the embryo, and there is the fact that the smaller egg, which is also the less yolky, develops as far as that of *Botryllus*.

The egg cleaves in typical unmodified ascidian manner, the eight-cell stage consisting of two tiers of four cells arranged in bilateral pattern as described by Conklin (1905) for *Styela*. Gastrulation takes place between the sixth and seventh cleavage. Subsequent development up to the tadpole stage, apart from the extensive intertissue spaces, is similar to that of *Botryllus* and has been adequately described by Garstang and Garstang (1928). These authors, however, describe hatching as a breaking through of the embryo into the atrium, but there seems to be little evidence of this. In fact the tadpole is relatively so large that it would in any case be a matter of great difficulty to escape in that way, and also the parent zooids are usually commencing regression at the time. Rupture of the external wall of the brood pouch liberating the tadpole directly into the common cloacal cavity appears to be the usual method employed (fig. 4 A–G).



TEXT-FIG. 4. Development.

A. Egg. B. Eight-cell stage. C. Gastrula (76-cell stage). D. Embryo with tail and separating ectodermal and endodermal layers. E. Late embryo with perforating protostigmata and young otolith. F. Late embryo showing oblique coiling of tail. G. Hatched tadpole from dorsal side showing branchial and atrial apertures, sensory vesicle, and ring of 8 ampullae around preoral lobe. H. Functional oozooid with protostigmata, basal ampullae, and first blastozooid. I. Oozooid absorbed, first blastozooid functional with 4 rows of definitive stigmata, and second blastozooid half-grown, common vascular system becoming established.

DEVELOPMENTAL TIMES

The whole developmental cycle, of bud and embryo, is a masterpiece of integrated timing. The course of growth of the developing bud, from its inception as a disk to its completion as a functioning zooid, follows a typical growth curve. The heart beats precociously but all other tissues come to function almost simultaneously after full size is attained. Eggs and sperm mature at this time. The growth of the egg ends with the completion of bud development.

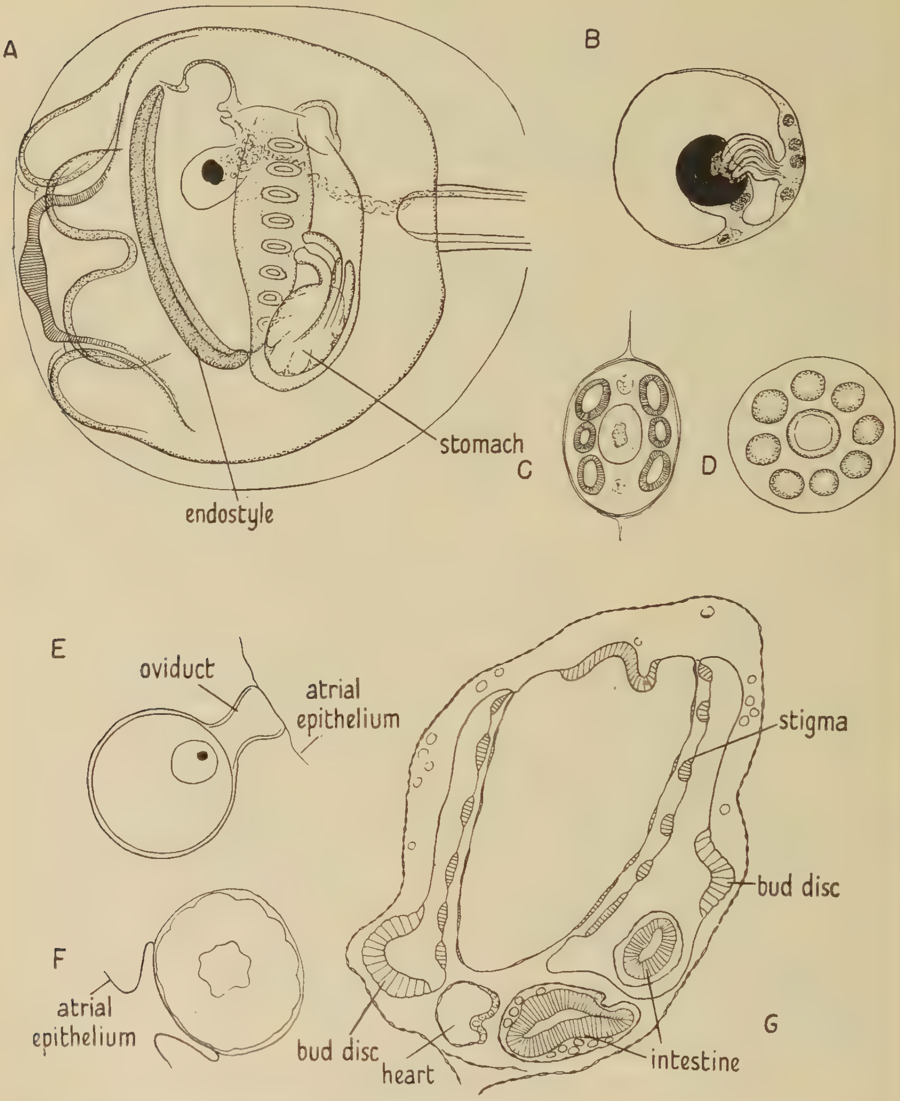
During the last phases of growth, that is, during the last day of bud development, the bud increases rapidly in size and at the same time the parent zooid is resorbed. Accordingly, all within a very few hours, the bud completes its development and growth, the parent zooid is resorbed, the eggs and sperm mature, and the development of the fertilized eggs begins. Usually throughout the colony, certainly within a system, all zooids perform in unison.

The functional zooids survive for a definite time at a given temperature, at the end of which they all resorb by tissue disintegration and autolysis into the colonial vascular system. Their life may be innately limited, but the determining factor in the end appears to be the rapid final expansion of the buds of the next generation, the vigour and nutritional demands of which giving them a priority that the relatively aged tissues of the older zooid generation cannot withstand. Thus the period of development of a bud that is in excess of the developmental period of its parental bud, lasting from the new bud inception to the completion of parental bud development, represents the duration of the parental form as an actively functioning organism.

On the other hand, the egg develops from the time it matures, coincident with the general completion of the zooid development, until it is liberated as an active tadpole when the zooid is about to be resorbed. Therefore, either the duration of egg development up to the tadpole stage is adapted to the survival period of the parental zooid, or the survival period is adapted to the length of the developmental period. Of the two alternatives, the first seems to be the more plausible. It is probably more feasible to modify the special developmental mechanisms of the egg that influence rate or duration of its development than to shift the time of bud initiation within the organization of a bud already in process of development. In any case, the mutual adaptation of sexual and non-sexual developmental processes is one of extremely close timing.

TADPOLE LARVA

In spite of the smaller, less yolky egg, and perhaps because of the complemental extra-embryonic nutrition, the tadpole of *Botrylloides* is very similar to that of *Botryllus*. The trunk region is relatively more expanded and is much like the trunk of the *Botryllus* 12 to 24 hours after the onset of metamorphosis. The tail retains the primary number of three bands of muscle-cells on each side of the notochord, and keeps its original orientation with the nerve-cord on the dorsal side. This is in keeping with all pleurogonid tadpoles



Text-fig. 5.

A. Lateral view of trunk of tadpole of *Botrylloides* showing preoral lobe ringed by epidermal ampullae, sensory vesicle with nerve-strand passing to dorsal side of tail, protostigmata, and digestive canal. B. Enlarged sensory vesicle showing single-celled otolith penetrated by light sensitive neurons to form photolith. C. Cross-section of tail showing 3 muscle-cells on each side. D. Anterior view of tadpole showing central lobe and ring of 8 ampullae. E. Ovum and oviduct of *Botryllus* for comparison. F. Gastrula of *Botryllus* supported by fold of atrial lining. G. Vertical section through young bud of *Botrylloides* showing atrial bud disks, heart, digestive canal, and pharyngeal and atrial components of developing gill slits (stigmata).

developing from large eggs and is in contrast to the 90° rotation typical of all large-egged entergonids, e.g. *Amaroucium*, *Perophora*, &c. The avoidance of rotation, as in related forms, is due to the oblique curving of the tail around the developing trunk so that torsion is not applied to the congested tail fin (fig. 4 F).

The sense organ is a 'photolith', an organ first described for *Botryllus* by Caswell Grave (1924) and so named by Garstang and Garstang (1928). It arises in the late development of the tadpole as a typical unicellular otolith. Later still, in fact almost belatedly, several adjacent neurosensory cells from the wall of the sensory vesicle grow in towards it and penetrate deeply into the mass of pigment. As in *Botryllus*, the tadpoles are both light- and gravity-sensitive in consequence (fig. 5 A, B).

Within the trunk the essential ascidian structure is well established, including endostyle, closed siphons, a vertical row of well-perforated protostigmata on each side, and a more or less differentiated digestive tube. Anteriorly there is a relatively wide and short stalk, the pre-oral lobe of Willey (1894), on the distal surface of which the three simple adhesive organs are formed. There is some doubt whether these organs are active in their normal function of attaching the tadpole when it first settles. The central stalk with its adhesive organs is surrounded by a ring of eight epidermal ampullae that in any case form the permanent organ of attachment, and the ampullae appear more able to assume this function at the time of settling of the tadpole than at the comparable time in *Botryllus* (fig. 5 D).

The active free-swimming period is on an average about 1 hour, with approximately 10 per cent. of the tadpoles remaining active as long as 12 hours. One day after the settling the siphons are prominent and the first blastozooid is visible as a small vesicle. At the end of the second day, the heart is beating, the siphons contractile, and the cilia of the gill slits just becoming active. As seen from the right side there are eight long protostigmata extending across the width of the branchial sac. The eight ampullae more or less radiate from beneath the base of the endostyle over the substratum.

The oozooid survives in an active condition for about a week, when it resorbs as the first blastozooid grows into functional activity. There is usually but one blastozooid of this generation, that of the right side, the one on the left usually starting to develop during metamorphosis but remaining abortive and disappearing. The ampullae of the oozooid survive the resorption process and link up with the ampullary vascular system of vessels established during succeeding blastozooid generations. The ampullae are independently contractile and aid in maintaining the colonial circulation (cp. Bancroft, 1899).

The first blastozooid differs from the oozoid mainly in having four rows of definitive stigmata on each side in place of the eight or nine undivided protostigmata. It is also without trace of gonads and has an atrial siphon similar to that of the oozooid. Subsequent blastozooid generations, when well nourished, produce both right and left buds, increase in number each generation, and increase in individual size as well; with the successive increases in individual size, the number of rows of stigmata increases from four to a dozen or more, the atrial siphons become first conical and then flute-mouthed, and orientate to establish star or ladder systems, while gonads develop, first male only, then later hermaphrodite (fig. 2 D–F, 4 H–I).

DISCUSSION

Botrylloides may be regarded as an evolutionary specialization of *Botryllus*. Whether this is true or not, the two genera are closely related and they can be compared in a strictly relative manner. Obviously, since the respective adult structure of zooid and colony is the product of certain basic developmental processes in each case, the differences exhibited by the two genera may be correlated with such differences in developmental activities.

In the case of bud development, the buds in both genera arise in the same relative positions at the same critical developmental stage of the parent bud, and involve the same tissues. There is the same general co-ordination of development of branchiae and gonad with initial bud size. In *Botrylloides*, however, there is a minor but significant difference in that the bud rudiment, in becoming a closed vesicle and thereafter, becomes relatively more elongated along its antero-posterior axis and relatively smaller in diameter. Three features appear to be correlated more or less closely with this growth difference. In conformity with the elongation of the developing branchial sac, there is space for more rows of stigmata to be laid down at the critical stage. The relative narrowness of the cylindrical form of the bud and zooid restricts somewhat the area from which bud disks develop, so that the maximum areas attained are relatively smaller than those finally produced in *Botryllus*. Consequently, mature *Botrylloides* zooids in their maximum state have one mature ovum and several small degenerate ova, comparable to the mature *Botryllus* zooid at its minimum condition (cp. Berrill, 1940), the maximum state in this form possessing four or five mature ova on each side. That is, the difference in the gonads of the two genera in a quantitative sense is a function of absolute size of buds at their inception. Thirdly, the difference in pattern of systems in the colony is due to differences in the shape of the atrial siphons of the zooids. That of *Botrylloides* appears to be the result of continued growth beyond the *Botryllus* condition involving a relative increase in growth rate of the transverse axis. This may in turn be related to the more basic differences in growth rates of the longitudinal and transverse axes of the developing buds taken as a whole.

Other intergeneric differences come under another category. The shift of the ovary from a position lateral to the testis, as in *Botryllus*, to one posterior to it in *Botrylloides* is apparently a difference in primary pattern not related to differences in dimensions or cell numbers, although in a more obscure way it may yet be an elongation effect. The development of a brood pouch as a local thickening of atrial epithelium at an early stage in bud development implies a local reactiveness and cellular differentiation absent in *Botryllus* and can only be an addition to the repertoire of cell specializations. Differences in fertilization procedure, if any, in early development of the egg, nature of embryo, its location and nutrition, and finally method of liberation may well be only consequences of the presence and nature of the primary brood-pouch epithelium as a local specialization of the atrial wall.

The suggestion is accordingly made that the various minor and major differences between the genera *Botrylloides* and *Botryllus* are the consequences of two basic differences, in the relative growth of the two primary axes of the bud, and a new cell specialization.

SUMMARY

The life-cycle of *Botrylloides* is described in comparison with that of *Botryllus* and, as far as possible, as a space-time continuum.

The origin, growth in size, form changes, and time relationships of the egg, embryo, and bud are described. Bud-development follows the same direct course as in *Botryllus*, but varying in size at each respective stage with the size of the initial bud disk. The gonad may be absent, male with or without rudimentary ova, or functional hermaphrodite according to the size of the bud disk and of the critical stage at which the various components of the gonad are segregated. Buds of each generation arise at the same precise stage of development of the parental buds. As each bud generation completes its development, the whole of the preceding generation is resorbed, and the newly matured and fertilized eggs of the new generation commence their development. The duration of egg development up to the formation of the active tadpole lasts until a few hours before the resorption of the parent zooid and escape to the common cloacal cavities is made directly by rupture of the parental body-wall. Eggs do not develop in the atrial chamber as in *Botryllus* but become enclosed in a special brood pouch, a unique structure arising early in the development of the bud and apparently independently of the presence of the gonads. Some form of extra-embryonic nutrition is evident and is supplied by the secretory lining of the placental brood pouch.

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Some Observations upon the Golgi Elements of the Anterior Pituitary Cells of Normal and Stilboestrol-treated Male Rats, using the Sudan Black Technique

BY

C. L. FOSTER

(*From the Dept. of Biology & Histology, Middlesex Hospital Medical School, London, W.1*)

With one Text-figure and one Plate

INTRODUCTION

THIS investigation was undertaken in order to discover whether the Golgi elements of anterior pituitary cells possessed the duplex structure described by Baker (1944) for cells of certain vertebrates and invertebrates, after fixation in formal-calcium chloride and staining in sudan black.

Pituitaries of normal mature male rats and comparable animals implanted with tablets of stilboestrol were used. The latter were studied for comparative purposes, since it is known that both synthetic and natural oestrogens activate the glandular cells of the anterior hypophysis, causing, among other changes, hypertrophy of the Golgi elements (Severinghaus, 1937; Foster, 1942).

METHODS

Six control and nine experimental rats with an average weight of about 150 gm. were used. The animals were killed by a blow on the neck, the experimental animals being killed at varying times after the subcutaneous implantation of a 10 mgm. tablet of stilboestrol.

The technique for demonstrating the lipoids of the Golgi zone with sudan black followed that described by Baker (1944). Since, in some instances, there was a tendency for a precipitation of the dye on the surface of the frozen sections, the following method was devised to overcome this difficulty.

The sections were first washed to remove the formal-calcium-cadmium chloride solution in which they had been stored and were then dipped in a 12 per cent. solution of gelatine, kept liquid in the 37° C. incubator. The slide was then lifted out and allowed to drain for half a minute, when the gelatine on the back was wiped off. The thinnish layer of gelatine on the section side of the slide was allowed to dry off until it became tacky, and was then coagulated by holding the slide, gelatine face downwards, beneath the surface of a dish of 90 per cent. alcohol. The section was then transferred to the filtered sudan black solution. The time necessary for adequate staining was, of course, found to be rather longer by this method and varied with the thickness of the gelatine film. In practice, the slide was periodically removed from the dye, rinsed in 50 per cent. alcohol, and examined under the microscope.

At the completion of staining, the slide was rinsed in 50 per cent. alcohol and the gelatine film was readily removed by immersion in warm water. After further washing in distilled water, the preparation was mounted in glychrogel (Cowdry, 1943).

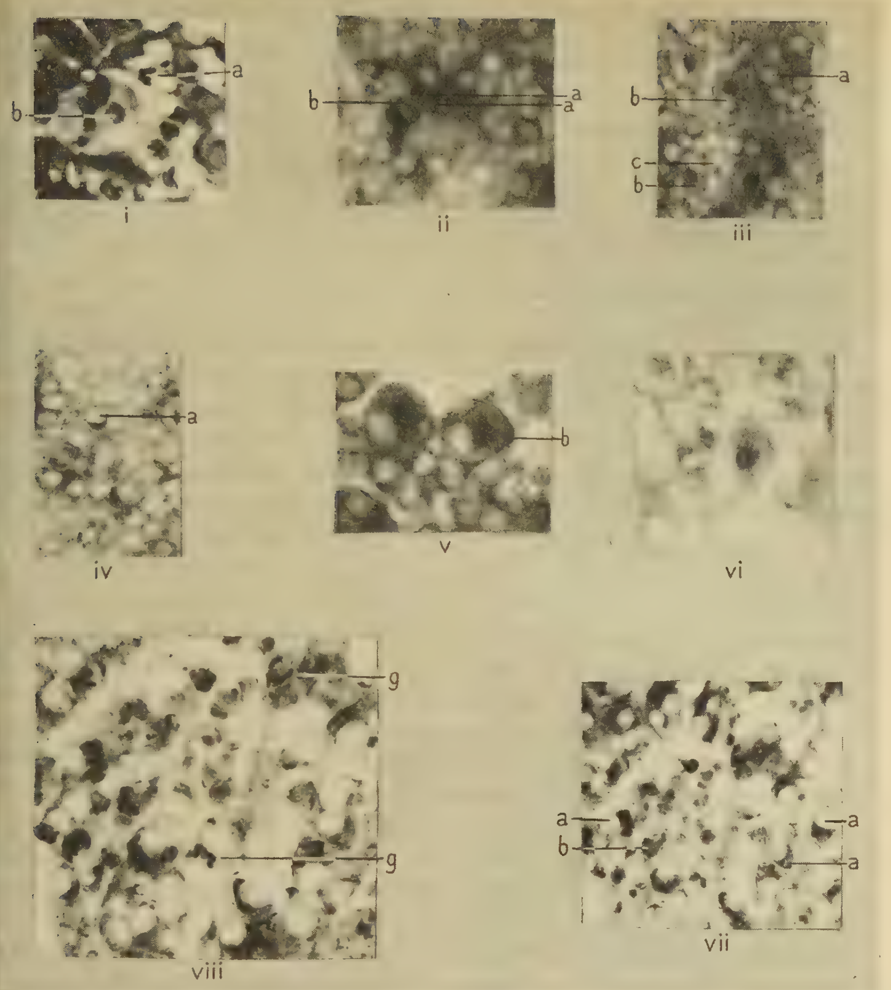
RESULTS

1. *Normal Animals*

(*a*) *The Position of the Golgi Zone.* In many instances the Golgi zone was clearly identifiable in the glandular cells of the pars distalis and details of its structure could be made out. This was particularly true of the basiphil cells, Golgi elements of which, as is well known, are revealed in Nassonov–Kolatchev preparations as fairly large spheroidal nets, generally situated at a distance from the nuclear membrane (cf. Text-fig. 1, *i* and Pl. 1, *i*, *iv*, *v*; Text-fig. 1, *ii–vi*). The details of the Golgi zones of the acidophil cells were, generally speaking, not so clearly defined, but the position and shape of the zone were almost exactly similar to those found in Nassonov–Kolatchev preparations; the zone was like a peruque capping the nuclear membrane. (Cf. Text-fig. 1, *i*, and Pl. 1, *iii*, *vii*, *viii*; Text-fig. 1, *ii*, *iii*, *iv*.) The Golgi elements in the small chromophobes were more difficult to make out, but in a number of instances it was possible to see that in some it had the shape and position characteristic of the acidophil type (Text-fig. 1, *iii*), while in others, its shape and position were such as are to be found in the basiphil cell (Pl. 1, *vi*). These observations were in agreement with those of Severinghaus (1936), who observed that in the mature gland, the two distinctive types of Golgi element characteristic of the acidophil and basiphil cells were present also in the chromophobes. Finally, the large chromophobes showed the same two forms of Golgi zone as was noted in the chromophils and small chromophobes —they are in fact, as has been shown by Severinghaus (1933), chromophils in the process of granule depletion.

Although the sections were not counterstained, very little difficulty was encountered in the identification of the cells types mentioned above, particularly as the preparations were studied in conjunction with stained Nassonov–Kolatchev material. The small chromophobes were the smallest elements of the cell population and were devoid of granules. The yellowish refractile granules of the acidophils were easily recognizable, and the basiphils were recognizable by means of their larger size, their indistinct granules, and characteristically placed Golgi bodies. The large chromophobes of the acidophil type were either partially or completely devoid of granules, as were those of the basiphil type. In the latter, this was reflected in a considerably reduced intensity of cytoplasmic staining with the sudan black.

(*b*) *The Structure of the Golgi Zone.* The Golgi zones of both acidophil and basiphil cells possessed three features in common and, in fact, differed



TEXT-FIG. 1.

i. Normal ♀. A, peruque-like Golgi element capping the nucleus of an acidophil cell; B, spheroidal Golgi zone of a basiphil cell. Nassonov–Kolatchev. Modified Mallory stain. ×650.

ii. Control ♂. A, Golgi zone of acidophil cell; B, Golgi element of basiphil cell. ×325.

iii. Control ♂. A, acidophil cell with Golgi zone immediately above and capping the nucleus; B, basiphil cells with characteristic Golgi elements, in which vacuoles are faintly discernible; C, acidophil type of small chromophobe with small Golgi zone in contact with lower edge of nucleus. ×325.

iv. Control ♂. A, acidophil cell with Golgi element consisting of granules of dense lipoid. ×325.

v. Control ♂. B, one of two large basiphil cells showing general form of Golgi zones, and their relation to nuclei. ×650.

vi. Control ♂. Curiously shaped basiphil cell in which vacuoles are faintly detectable in the Golgi zone, the central region of which is occupied by diffuse lipoid. ×650.

ii. Experimental ♂. Stilboestrol 32 days. A, acidophils with hypertrophied Golgi zones in which the diffuse lipoid and dense lipoid material is readily seen; B, hypertrophic Golgi area of a basiphil showing similar features to the above. ×325.

iii. Experimental ♂. As above. G, Golgi elements showing rows of enlarged vacuoles enveloped in dense lipoid material. This is a more highly magnified area from the centre of *vii.* ×650.

from one another only in the two respects already mentioned—(*a*) in shape, and (*b*) position in relation to the nucleus.

The three features were:

1. A zone of diffuse, less deeply staining sudanophil material, embedded in which were:
2. Small clear vacuoles, and
3. Strongly sudanophil elements.

The position and shape of the diffuse sudanophil material were primarily responsible for determining the morphology of the Golgi zone as a whole. That is to say, the difference between the acidophil and basiphil types was directly related to differences in the size, shape, and position of the diffuse sudanophil material and not to the nature of the vacuoles and the strongly sudanophil inclusions (cf. Pl. 1, *i* and *vii*). In some cases, particularly in small cells, where the diffuse sudanophil material could not be readily made out, the distribution of the strongly sudanophil material was sufficient to characterize the type of Golgi zone (Pl. 1, *iii*).

The vacuoles, although normally small, were variable in size (Pl. 1, *viii*), and, in occasional instances, were to be seen outside the zone of diffuse sudanophil material (Pl. 1, *iv*). The relationship between these vacuoles and the strongly sudanophil elements was not always clear-cut. Sometimes, as in Pl. 1, *iv*, *v*, *viii*, some of the vacuoles appeared to be unconnected with the sudanophil elements, but this may perhaps have been due to the extreme thinness of any sudanophil film investing them.

In those instances where there was clearly a relationship between the intensely sudanophil particles and the clear vacuoles, the former appeared either as small granules of which one and sometimes two were seen to be in contact with the edge of a vacuole (Pl. 1, *iv*, *v*, *vii*) or else as sudanophil arcs capping single vacuoles (Pl. 1, *ii*, *viii*). There was evidence in some instances that these 'caps' were approximately crescentic in form (Pl. 1, *viii*), but in others they appeared to be of a uniform thickness (Pl. 1, *iv*, *v*). A few examples of vacuoles apparently completely invested with a sudanophil film were observed (Pl. 1, *viii*).

In some cells there was a high proportion of small, strongly sudanophil, granule-like bodies of variable size, apparently unrelated to any external or internal vacuole. Pl. 1, *iii* shows a rather small acidophil type cell whose Golgi zone consisted entirely of these apparently solid granules.

Thus, in these Golgi bodies, in addition to the region of diffuse sudanophil material usually present, there were observed to be:

1. Vacuoles apparently unrelated to the strongly sudanophil bodies;
2. Strongly sudanophil elements apparently unrelated to vacuoles; and
3. Vacuoles associated with caps or granules of strongly sudanophil material.

2. *Experimental Animals*

(*a*) *The Position of the Golgi Zone.* Any alteration in the position of the Golgi zone was clearly due to the generalized hypertrophy of the whole area. This hypertrophy in response to oestrogens is already well known from observations based upon the study of material prepared by the Nassonov–Kolatchev method (Severinghaus, 1937). When the hypertrophy was considerable, there was a tendency for the Golgi region of the basiphil cells to encroach upon the nuclear region and make contact with the nuclear membrane (Pl. 1, *xi*), and this resulted in a somewhat acidophil-like morphology (cf. Pl. 1, *xi* and *viii*). In neither type of cell, however, was there evidence of any fundamental shift in position, and only occasionally was difficulty encountered in deciding the type of cell under observation.

(*b*) *The Structure of the Golgi Zone.* The three features mentioned as being characteristic of the Golgi regions of normal cells were again readily seen in the experimental animals, and, generally speaking, the differences between the two were quantitative rather than qualitative, although certain qualitative differences were observed.

Even after a relatively short period of implantation with stilboestrol there was quite a marked increase in the amount of diffuse sudanophil material, relative to the cytoplasmic volume (cf. Pl. 1, *v* and *xi*; *vi* and *x*), and with more prolonged treatment the increase was unmistakable (cf. Pl. 1, *viii* and *xiii*; Text-fig. 1, *ii* and *vii*). Concurrently, the intensity of the staining of the diffuse material also increased, but this may perhaps have been due to a closer packing of the sudanophil particles and hence may have been a quantitative and not a qualitative change. The Golgi elements of all cell types were similarly affected, but since one of the characteristic effects of prolonged treatment with oestrogens is a granule depletion in the chromophil cell (Severinghaus, 1937), the cells observed in those glands subjected to a more prolonged treatment were commonly extremely degranulated—they were in fact large chromophobes.

The effect of the oestrogen upon the vacuoles and strongly sudanophil elements was, after fairly prolonged treatment, quite clear-cut. First, there was an increase in both the amount of vacuolation (cf. Pl. 1, *vii* and *xii*) and the amount of strongly sudanophil material (cf. Pl. 1, *v* and *xi*), although the latter was not always so marked. As in the controls, there were vacuoles apparently unrelated to sudanophil elements and there were granular sudanophil elements unassociated with any external or resolvable internal vacuole (Pl. 1, *ix, xii, xiv*). Secondly, there was a qualitative change resulting from the coalescences of vacuoles. This, in its extreme form, is shown in Pl. 1, *xv* and Text-fig. 1, *viii*, where vacuolar chains of large size were produced; in other instances, the evidence for vacuole fusion was based on the occurrence of vacuoles considerably above the normal size (Pl. 1, *xiii, xv*). These were often associated with several sudanophil granules and crescents, instead of the more usual one or two seen in the controls. The sudanophil elements were generally of the same type as in the controls, that is to say: crescents (Pl. 1, *ix*, *x*, *xi*), caps of apparently uniform thickness (Pl. 1, *xi*) and occasionally continuous films (Pl. 1, *ix*). Appearances such as those shown in Pl. 1, *xiv*, where the sudanophil material was elliptical in shape, suggested that ring-like configurations were also present.

The results described above show that the essential structural features of the Golgi body were maintained after fairly prolonged treatment with stilboestrol. The hypertrophy produced by oestrogens was due to (1) an increase in the amount of sudanophil material of both types, and (2) an increase in the amount of vacuolation.

Discussion

Since sudan black is known to have a high and apparently specific solubility in lipoids (Baker, 1944) it can be justifiably assumed that the sudanophil material of the Golgi elements just described consists, at all events in part, of lipoid substances. The Golgi zones of the cells of normal and experimental animals thus consist of regions containing diffuse lipoids in which are embedded three sorts of structures: (*a*) clear vacuoles, (*b*) lipoid-containing granules, and (*c*) clear vacuoles partially or completely invested with dense lipoid material. It is reasonable to believe, for reasons indicated by Baker (1944), that the technique preserves these structures in a form not greatly dissimilar to that obtaining in the living cell and, in any case, it seems improbable that any distortion produced would exceed that caused, for example, by the Nassonov–Kolatchev procedure. There is in fact a very close similarity between the morphology of the Golgi zone as revealed by the latter method, and that demonstrable by the use of sudan black.

Severinghaus (1932), using the Nassonov technique, showed that two sorts of Golgi element, associated respectively with acidophils and basiphils, were present in the anterior lobe of the rat pituitary. The result of the present investigation supports his observations. The fact that two distinctive forms of Golgi element regularly exist in this way in the two sorts of chromophil cell (Severinghaus, 1933, 1937) of the rat, the guinea-pig (Kirkman, 1937), and perhaps less markedly in the cat (Dawson, 1946) is of theoretical interest, since it suggests that the Golgi zone, in spite of its lability, must be linked to the 'cytoskeleton'. In the rat, the characteristic Golgi zone morphology of the two types of chromophil cell is retained after treatment with oestrogens, except where the dosage is particularly heavy; but the evidence again suggests that the pattern of the Golgi zone is related to the ultrastructure of the cytoplasm.

The Golgi material of the anterior pituitary cells of the rat, as revealed by the Nassonov–Kolatchev technique, for example, consists of a network of osmiophil threads and granules associated, according to observers such as Severinghaus (1937) and Ayers (1941), with clear vesicles or vacuoles. The latter described such vesicles as having three different relations within the cell: (1) the smallest, appearing as 'clear, tiny ovoid spaces incorporated in the strands of the Golgi net', (2) '. . . slightly larger vesicles eccentrically located so that only a thin rim covers the outer side'—such vesicles being up to three times the size of the previous type—and (3) vesicles present in the cytoplasm, some of which 'have a bit of osmiophil material adherent'. There can be little doubt that these vesicles are similar to the vacuoles observed in the sudan black preparations, and it seems likely that the stranded osmiophil material in which they are embedded, or with which they are associated, corresponds partly to the diffuse and partly to the denser lipoid already described. Also, cytoplasmic vacuoles associated with dense lipoid material were occasionally observed in the sudan black preparations (Pl. 1, *iv*).

The 'classical' reticulate appearance of the Golgi elements in pituitary cells as seen after the Nassonov and similar techniques may well be due to the shrinkage and distortion of the complex revealed by the sudan black method. Some evidence in support of this notion has been brought forward by Worley (1943 *a* and *b*, 1944), who showed (1) that structures comparable to the Golgi elements in regard to their position in the cell and their relation to other cytoplasmic parts (e.g. secretion droplets, &c.) were, in various invertebrate and vertebrate cells, stainable supravitally with methylene blue, and (2) that shrinkage induced by dessication in many instances resulted in a picture very similar to that normally produced by the customary osmium tetroxide impregnation techniques. In the living pancreas, for example, there was a progressive distortion of the stained spherical bodies from which, after a time, there extended thread-like processes whose anastomoses with one another produced a network.

Furthermore, the observations of Worley are in support of those of Baker (1944) and the writer, in that they provide evidence for a duplex structure of the Golgi element—a concept very extensively developed by the investigations of Hirsch (1939). Worley showed that the Golgi vesicles in the cells he studied regularly progressed from a small methylene-blue-stained granular stage to one where the granule had enlarged and differentiated into a clear vacuole with a chromophil pellicle of varying thickness. The vacuole was the site of production of the secretion product, liberation of which was associated with the fragmentation of the chromophil cortex. It seems probable that these two parts of the vesicle correspond to Hirsch's 'Internum' and 'Externum', and the writer believes that this correspondence may be extended to the vacuoles and the associated lipoid elements of the Golgi bodies of the rat anterior pituitary. It is possible, moreover, that the dense sudanophil structures unassociated with vacuoles are equivalent to Hirsch's 'Presubstanz', from which the corticated vacuoles of the fully developed Golgi zone are probably derived. Baker (1944) has suggested the extension of the term 'Externum' so as to include the diffuse as well as the dense lipoid material.

The hypertrophic effect of oestrogens upon the Golgi element of anterior pituitary cells has been clearly established (Severinghaus, 1937), but sudan black because of its high solubility in lipoids gives more precise information about the details of this hypertrophy. Severinghaus (loc. cit.) has described an increase in the vacuolation of the Golgi zone after oestrone injections and the method used in this investigation showed that not only may there be an increase in the number of vacuoles, but, after prolonged treatment with stilboestrol, coalescence of these vacuoles occurred (Pl. 1, *xv*). There appeared also to be an increase in the amount of both diffuse and dense lipoid material and in some instances coalescence of the former to produce sudanophil strands. Such changes as these, considered in relation to changes known to occur in the Golgi bodies of other endocrine as well as exocrine glands (Bowen, 1929; Kirkman and Severinghaus, 1938; Foster, 1942; Bourne, 1942), seem to indicate a state of heightened secretory activity. Finally, as has been suggested before (Severinghaus, 1936; Foster, 1942*a*), it is thought likely that the physiological inhibition produced by oestrogens is due either to over-activation of the secretory mechanism (of which the Golgi element is almost certainly a part) or to an ultimate interference with some stage in the metabolism of the hormonal secretion product.

SUMMARY

1. It was found possible to demonstrate the Golgi zones of the cells of the anterior pituitary (pars distalis) of the rat with sudan black. The position and general morphology of the Golgi bodies closely resembled that revealed by the standard osmium tetroxide techniques.

2. The Golgi zone was generally found to contain three sorts of element: (*a*) diffuse sudanophil material in which were embedded: (*b*) clear vacuoles and (*c*) strongly sudanophil bodies. The latter were often associated with the surfaces of the vacuoles, when they were in the form of (1) crescentic caps, (2) partial or complete rims of apparently uniform thickness, or (3) small granules. Sometimes the strongly sudanophil bodies were granular and unassociated with vacuoles; there were also vacuoles with no demonstrable lipoid material in association with their surfaces.

3. The effect of stilboestrol was to cause hypertrophy of the Golgi zones and this was associated with (*a*) an increase in volume of the region of diffuse lipoids, (*b*) an increase in vacuolation, and (*c*) a rather more variable increase in the amount of strongly sudanophil material. These changes would appear to indicate a state of heightened secretory activity.

4. The strong affinity of the Golgi area for sudan black suggests that it consists, at any rate in part, of lipoids.

I wish to express my thanks to my colleague Mr. W. F. Floyd for the invaluable help he has given with the photomicrography. This investigation was carried out with the assistance of a grant from the Thomas Smythe Hughes Medical Research Fund of the University of London.

EXPLANATION OF PLATE 1.

Figs. *i–xv* are from camera lucida drawings.

i. Control ♂. Basiphil cell showing general form of Golgi zone.

ii. Control ♂. Acidophil cell. Upper focal plane, showing diffuse lipoid and vacuoles with caps containing dense lipoid.

iii. Control ♂. Acidophil cell with peruque-like Golgi zone consisting of dense lipoid particles.

iv. Control ♂. Large basiphil cell with vacuoles outside area of diffuse lipoid, and vacuoles unassociated with dense lipoid.

v. Control ♂. Basiphil cell, showing diffuse lipoid, and vacuoles associated with incomplete dense lipoid rims or granules.

vi. Control ♂. Basiphil type of small chromophobe showing Golgi zone.

vii and *viii.* Control ♂. Acidophil cells showing vacuoles of varying size with dense lipoid rims and crescents, and also isolated granules containing dense lipoid.

ix. Experimental ♂. Stilboestrol 6 days. Acidophil cell, showing vacuoles with complete investments of dense lipoid.

x. Experimental ♂. Stilboestrol 19 days. Acidophil cell showing enlarged area of diffuse lipoid and numerous vacuoles of varying size, with crescents, rims, and granules of dense lipoid.

xi. Experimental ♂. Stilboestrol 19 days. Basiphil cell showing enlarged area of diffuse lipoid, vacuoles of varying size, some unassociated with dense lipoid.

xii. Experimental ♂. Stilboestrol 19 days. Acidophil cell showing vacuoles outside zone of diffuse lipoid.

xiii. Experimental ♂. Stilboestrol 49 days. Acidophil cell showing greatly increased zone of diffuse lipoid.

xiv. Experimental ♂. Stilboestrol 43 days. Acidophil cell showing two markedly elliptical vacuoles with rims of dense lipoid.

xv. Experimental ♂. Stilboestrol 56 days. Basiphil cell showing hypertrophy and partial fusion of the vacuoles and coalescence of the dense lipoid material to form strands.

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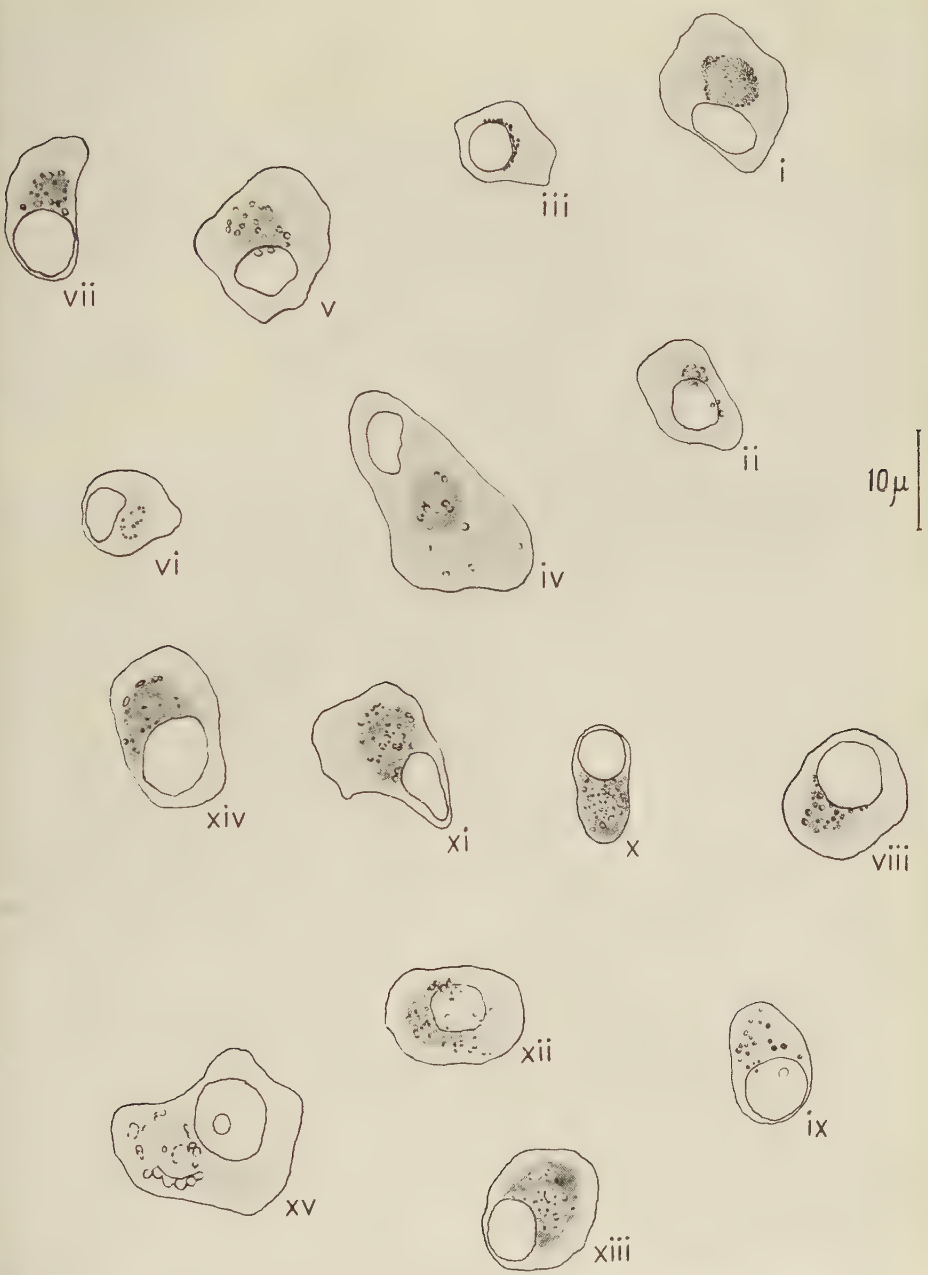

C. L. FOSTER.—PLATE I

A Cytological Study of the Alimentary Tract of the Domestic Fowl (*Gallus domesticus*)

BY

K. S. CHODNIK, Ph.D., M.R.C.V.S.

(*From the Department of Zoology, University of Edinburgh*)

With two Plates and four Text-figures

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INTRODUCTION

THE work recorded in this paper was undertaken in order to investigate the morphological changes which the cytoplasmic components undergo during different physiological phases induced by fasting and food stimuli, and to study the role of the Golgi material and mitochondria in secretory phenomena. The examination of the proventriculus, gizzard, and intestine yielded information on the cytology of gastric and intestinal epithelia which does not seem to have been previously recorded.

While there are extensive histological studies on the alimentary tract of the fowl (Clara, 1926–7; Calhoun, 1933) there are few published accounts of its cytology. Workers in this field, owing to the suitability of the material, concentrated mainly on cytoplasmic changes during embryonic life. Brief cytological accounts of the mitochondria and Golgi material of various cells which compose the lining of the intestine of birds are given by Clara (1926–7). Argeseanu and May (1938), in their work on the intestinal epithelium of domestic fowl, outline, as the principal aim of their work, similarities and differences which exist between the cell components at various stages of development. Their observations on the Golgi material are based exclusively on rather unreliable silver nitrate impregnations. Hibbard (1942) describes the changes in form and position of the Golgi material in the proventriculus and gizzard of the domestic fowl throughout the stages of embryonic development. None of the papers quoted contains a complete survey of all the cytoplasmic structures in any part of the alimentary tract investigated by the present writer.

There are numerous papers dealing with the cellular changes which take place during digestion and absorption; these include a wide variety of material ranging from the simplest protozoa to the complicated intestinal epithelium of mammals. Some of these works will be discussed in a later part of this paper.

MATERIAL AND METHODS

The material used in the present work consisted of samples taken from different parts of the intestinal tract, namely, proventriculus, gizzard, duodenum, ileum, caeca, and rectum of the domestic fowl (*Gallus domesticus*).

Material was obtained from young chickens (Brown Leghorn) aged between 3 weeks and 3 months old. Chickens, battery reared, of uniform breed and management, and free from disease, were chosen. All specimens were killed and small pieces of the tissues immediately dissected out and placed in one of the fixing fluids.

At least two birds were used to investigate each physiological phase. When the secretory cells were found to be active, and marked morphological changes observed, additional specimens were used as a check to avoid mistakes due to technical errors. At first, birds were chosen after 24 hours fasting with free access to water. Observations on all subsequent phases were undertaken at various times after feeding. Each feeding experiment was preceded by 24 hours fast, after which food was given freely. Specimens were killed half an hour after feeding and at hourly intervals terminating 6 hours after the meal. The last specimens examined were those with constant access to food without a previous fast. Generally, at least two different cytological methods were used simultaneously both for Golgi and mitochondrial preparations. For mitochondria the best results were obtained with Regaud's technique, the tissues being fixed in a refrigerator. Meves's mixture, according to the following formula, gave the next best results: 0·5 per cent. chromic acid in 1 per cent. sodium chloride solution, 15 c.c.; 2 per cent. osmium tetroxide, 3 c.c. The fixing fluid was changed after 4–5 hours, and further fixation continued for 48 hours. Some other methods were also tried. In order to demonstrate the Golgi material, Mann-Kopsch technique was used according to the original formula or with modifications. The methods of Kolatchev and

Sjöval and the silver methods of Aoyama and Da Fano (in spite of less constant results with the latter) proved useful controls. The material was embedded in paraffin. Sections were cut at 5 μ and 7 μ in thickness. For general histological purposes Bouin and Zenker-formalin proved the most useful fixatives. Golgi preparations as a rule were mounted unstained or in a few cases counter-stained with Altmann's acid fuchsin and differentiated in 90 per cent. alcohol instead of in picric acid. Staining methods for mitochondria consisted of Heidenhain iron haematoxylin, Regaud's haematoxylin, always optimal for the Regaud's preparations, Altmann's acid fuchsin, and Bensley's acid fuchsin light green. Southgate's mucicarmine was used for the demonstration of mucus.

The various fixatives used did not give equally good results with different parts of the alimentary tract. In the case of the stomach, fixatives containing osmium tetroxide revealed many more secretory granules than the other techniques employed; consequently these fixatives are of little use for the study of the mitochondria of zymogenic cells. Observations on the Golgi material of the zymogenic cells were based exclusively on silver impregnated material (Aoyama and Da Fano), as all attempts to obtain good osmic preparations of these cells were unsuccessful. Aoyama's method gave the best results when the tissue was fixed for 4–5 hours. As regards the intestine, the best results were obtained by taking samples from the part situated a short distance above and below the line marked by the descending bowel contents. This routine was carried out in all fed specimens.

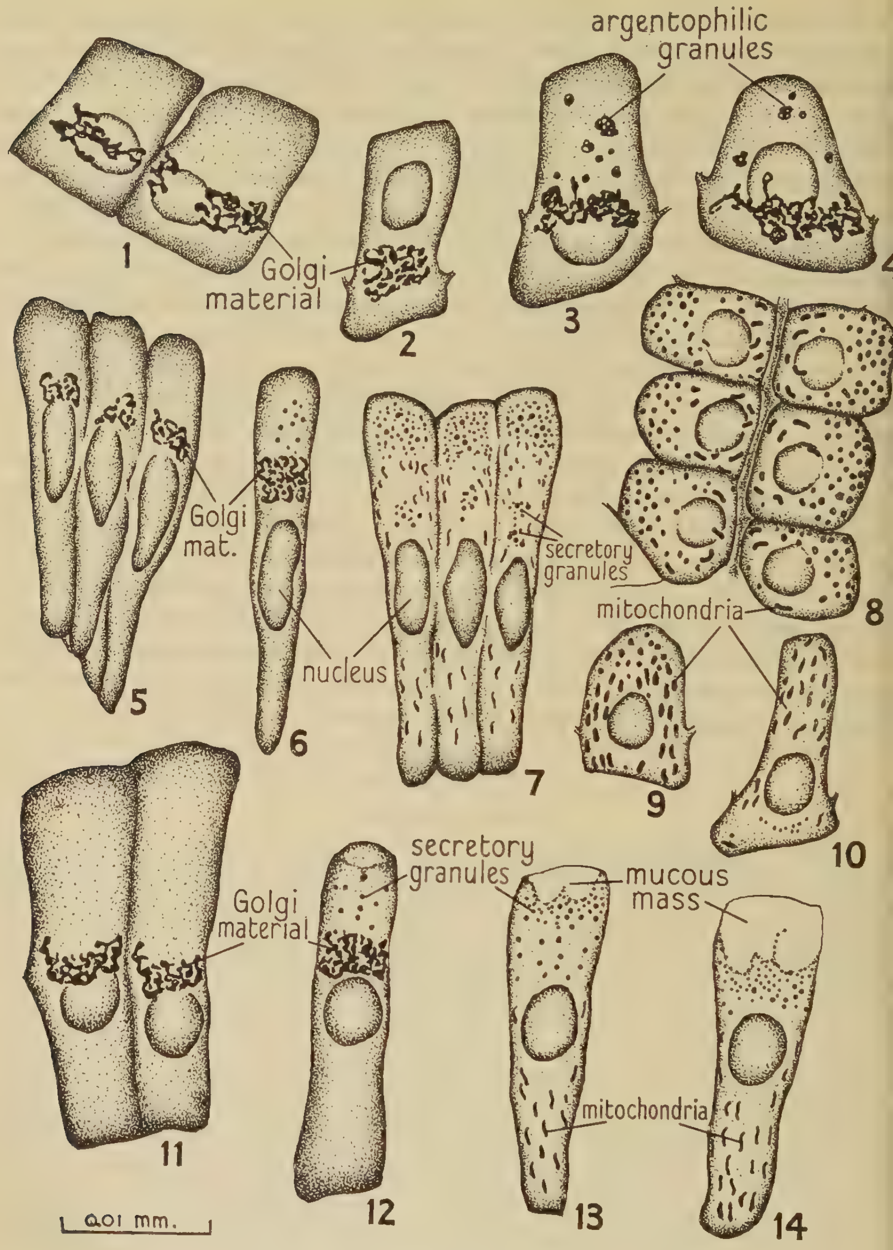
OBSERVATIONS

Proventriculus

Highly differentiated groups of cells compose the lining of the simple tubular glands and the surface of the gastric lumen. It is therefore necessary to give a separate description of the different types of gastric cells. The zymogenic cells in the tubular glands (Text-fig. 1, figs. 1–4; Pl. I, fig. 16) differ markedly from the superficial columnar epithelium (Text-fig. 1, figs. 5–7); the latter are very similar in morphological outline to those of the intestinal epithelium. Over the short distance which separates the mucous neck cells from the zymogenic cells which line the glandular crypts are scattered cells which cytologically resemble the outer epithelium and, in gross morphological outline, the zymogenic cells. These cells more closely resemble the chief neck cells described by Okanishi (1933) than the parietal cells of mammals. These transitional neck cells have not been identified during the present investigations in the deeper parts of the glandular crypts, and no reference to them was found in the accessible literature on the avian stomach.

Zymogenic Cells

Various names are used to describe the zymogenic cells, e.g. the main cells of the gastric glands (Eklöf, 1914), oxyntic cells (Chinese workers), and zymogenic cells (British workers).



Text-fig. 1.

All figures from proventriculus.

Figs. 1–4 from Aoyama preparations; 5–6 and 11–12 from Kolatchev preparations; Figs. 7–10 and 13–14 from Regaud preparations.

Fig. 1. Zymogenic cell showing Golgi material; after 24 hours' fast.

Fig. 2. Zymogenic cell 1 hour after feeding, cell elongated, apparent reversed polarity of Golgi material.

The zymogenic cells are arranged in a single layer which lines the simple tubular glands along their whole length. Their shape varies, depending on the degree of functional activity, from low cuboidal to elongated columnar cells. In longitudinal sections of the gland these cells are arranged obliquely with their long axis directed slightly towards the opening of the gland. They are arranged in rows in such a way that their distal half, or more than two-thirds of the cell, is not in contact with the corresponding part of the neighbouring cells; thus straight canaliculi are formed between the lateral sides of neighbouring cells (Text-fig. 1, fig. 1, Pl. I, fig. 16–Y). This gives longitudinal sections a serrated edge formed by the distal parts of the cells bulging into the lumen of the glandular crypt.

The nucleus is spherical to ovoid in shape and its position depends on the functional stage of the cell; it may lie close to the basal membrane or towards the central region. In all phases of fasting and digestion, most of these cells are densely packed with large, spherical, secretory granules. The number of granules increases considerably during fasting, and in the later stages they extend from the end next to the lumen to the basal part of the cell where they form a mass of closely packed granules which greatly hinders cytological observations. After 24 hours' fast, when there is a maximum accumulation of secretory granules, the cells assume a more cuboidal form with smooth, rounded outlines. The nucleus is spherical and is situated close to the basal membrane. The intercellular canaliculi are difficult to follow, as the lateral cell membranes, under the pressure of accumulated secretion, are brought into proximity with their neighbours (Text-fig. 1, fig. 8). Commencing half an hour after the ingestion of food the number of secretory granules decreases considerably and the outline and shape of the cell become altered. The cell becomes thinner, elongates, and assumes an angular outline. The nucleus moves closer to the central region of the cell and assumes an ovoid shape (Text-fig. 1, fig. 2). The intercellular canaliculi now appear as incisions or long narrow clefts wedged between the cells; this is due to the contraction of the lateral cell borders. The evacuation of the cells, which starts with the intake of food, increases markedly until 3 hours after a meal. In sections prepared for the study of the mitochondria, unstained vacuoles appear in

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Figs. 3 and 4. Zymogenic cells 2 hours after feeding; argentophilic granules present in cytoplasm.

Fig. 5. Cell of surface epithelium showing Golgi material; after 24 hours' fast.

Fig. 6. Cell of surface epithelium, 1 hour after feeding, Golgi material enlarged; secretory granules seen above it.

Fig. 7. Cell of surface epithelium showing mitochondria and secretory granules.

Fig. 8. Zymogenic cells showing mitochondria and secretory granules; after 24 hours' fast.

Figs. 9 and 10. Zymogenic cells 1 hour after feeding; showing mitochondria and secretory granules below the nucleus in fig. 10.

Fig. 11. Mucous neck cells showing Golgi material; after 24 hours' fast.

Fig. 12. Mucous neck cell 1 hour after feeding; Golgi material enlarged and secretory granules seen above it.

Figs. 13 and 14. Mucous neck cells 1 hour after feeding; showing mitochondria, secretory granules, and formed mucous mass.

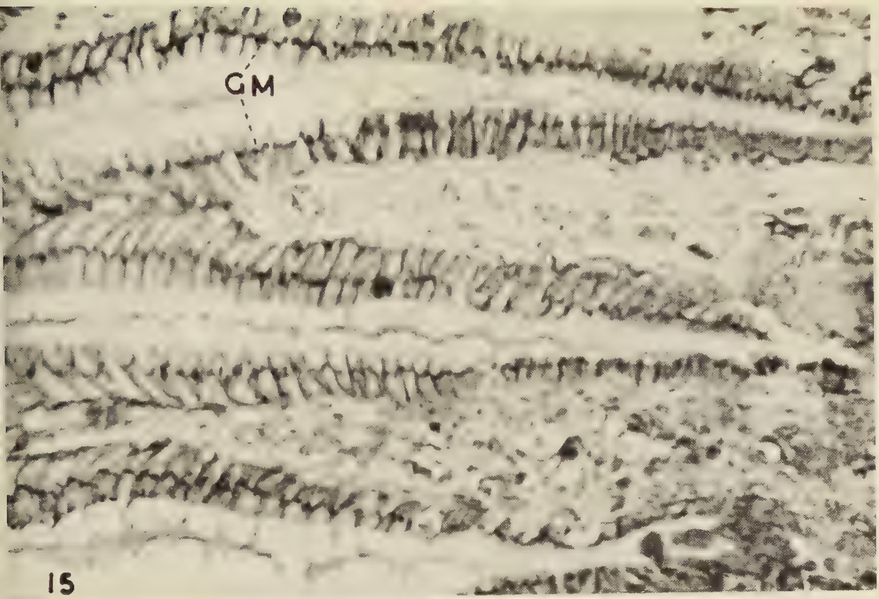
the place of secretory granules. Three hours after feeding the cells begin to refill with secretory granules, and return to their former shape. The number of granules gradually increases, and 6 hours after the intake of food reaches the level usually observed in birds with constant access to food, which is never as great as in fasting specimens.

It must be noted that only a certain number of cells is involved in the secretory process at the same time, and that each cell appears to act as an independent unit. During the first hours of digestion, cells situated close together often show marked differences in shape and functional stages. Cells at the bottom of the glandular crypts appear to be the least involved in secretion and contain fewer secretory granules than any of the other cells.

Golgi material. The Golgi material in zymogenic cells was studied exclusively in material impregnated with silver (Aoyama and Da Fano). Certainly these methods are much less delicate than osmic methods, but when carefully handled they provide much valuable information. No great difficulties were encountered with silver methods and the Golgi material was shown in cells along the whole length of the glandular canal. Cells in the middle of the glandular tubule are more difficult to impregnate than those nearer the bottom and neck of the crypt; this is due to the large number of secretory granules present in the cells of the middle region.

The Golgi material in zymogenic cells consists of thick filaments, twisted and joined in a more or less compact manner. It always lies in the basal part of the cell exactly at the level of the bottom of the intercellular canaliculi (Text-fig. 1, fig. 1; Pl. I, fig. 16). After 24 hours' fast, when the cells are packed with secretory granules and the nucleus is close to the basal membrane, the Golgi material surrounds the nucleus to form an equatorial belt (Text-fig. 1, fig. 1). The elements of the Golgi material seem to be closer together than in actively secreting cells. With the decrease in the number of secretory granules, subsequent to feeding, the cell elongates and the nucleus moves towards the central region. The Golgi material does not move with the nucleus and thus gives what students of the theory of polarity would describe as 'reversed polarity'. At this phase there is a considerable loosening of the Golgi elements towards both poles of the cell. The Golgi material, however, never moves from its original level (Text-fig. 1, fig. 2; Pl. I, fig. 16–x.)

In the next phase, elongate cells, with Golgi material situated below the nucleus, show changes which strongly suggest that secretory activity has begun. Small clusters of argentophilic granules are present above the Golgi material in the supranuclear zone (Text-fig. 1, figs. 3–4). In deeply impregnated material the granules are blackened and appear as a uniform mass, but in lightly impregnated material the clusters of granules are clearly shown, and the faintly brown outline of each granule is visible. This argentophilic material was observed in many cells 1–2 hours subsequent to feeding; after that time they were less frequently seen and were seldom observed 3 hours after feeding. It is probable that these granules migrate from the Golgi zone during increased cellular activity, and represent a stage in the formation of



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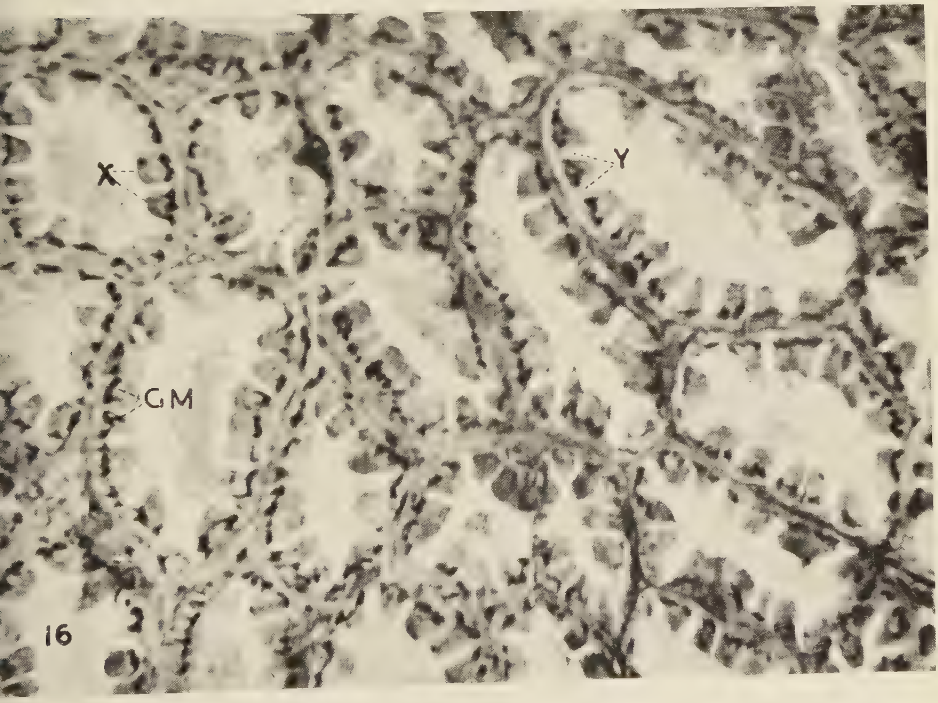

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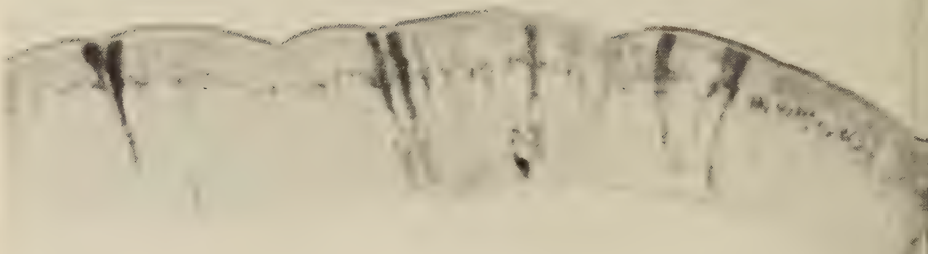
K. S. CHODNIK.—PLATE I



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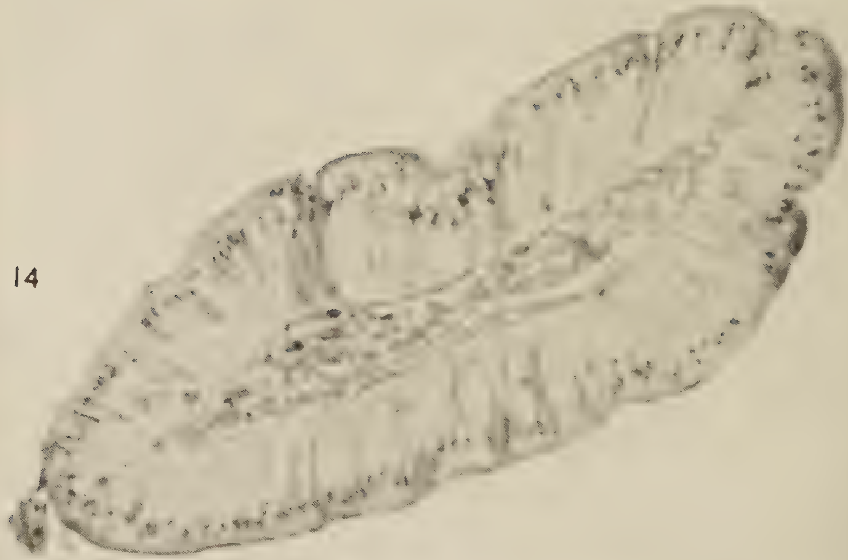

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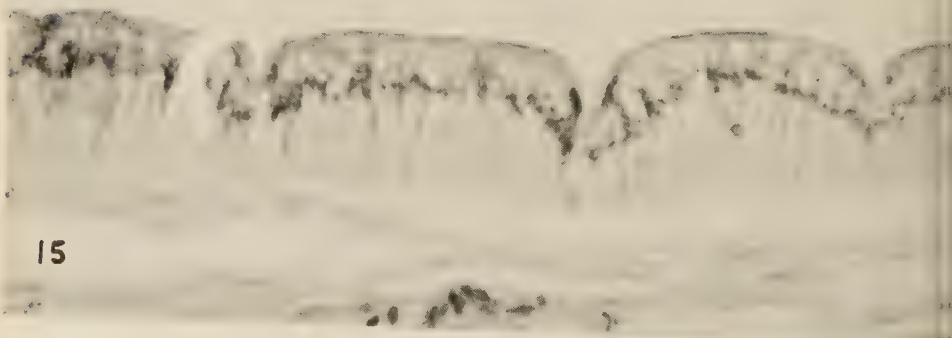

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K. S. CHODNIK.—PLATE II

the secretion granules. As they are visible for a short time after feeding (1–2 hours) and are not present at any other phase, it is probable that a rapid increase in secretory activity leads to the freeing of products which are not yet fully formed and possess the argentophilic properties of the Golgi material itself, and that their transformation into granules of secretion takes place in the cytoplasm. Owing to the coarse outlines of the Golgi material (silver methods), it is impossible to identify secretory granules amongst the Golgi material. The earliest secretory granules are visible in mitochondrial preparations and are restricted to the Golgi field (Text-fig. 1, fig. 10).

Present observations on zymogenic cells support the view that the situation of the Golgi material, at least in these cells, is not influenced by the relative position of the nucleus. The apparent reversal of polarity, which has aroused much discussion, is of short duration and is produced by a free movement of the nucleus and not of the Golgi material.

The location of the earliest secretory granules in the Golgi field, the presence of clusters of argentophilic material which migrates from the Golgi zone during the later phase of secretion, and the changes in the form of the Golgi material indicate that the Golgi material participates in secretory activity.

Mitochondria. As Regaud's haematoxylin tends to stain mitochondria only, clear pictures of the mitochondria were obtained in zymogenic cells from fasting and fed specimens prepared by this method.

Short rod-shaped mitochondria are numerous; in addition a few long thick filaments, of equal diameter, are present. The small number of granules observed would suggest that they are cross-sections of rods and filaments. A few swellings situated at intervals along the mitochondria are sometimes visible. In cells densely filled with granules the majority of the mitochondria are situated in the basal half of the cell, but a few are also dispersed in the supranuclear zone and are visible between the secretory granules (Text-fig. 1, fig. 8). At this phase the mitochondria are arranged more or less at random without a special polar orientation.

At the time when the elongate cells enter upon secretory activity, the mitochondria become distributed throughout the cytoplasm, but the majority are in the supranuclear zone. Polar orientation is strongly marked by the arrangement of the rods parallel to the long axis of the cell (Text-fig. 1, figs. 9–10). In the early phases, very small and deeply stained granules (Regaud's haematoxylin) are observed below the nucleus in the region occupied by the Golgi material (Text-fig. 1, fig. 10). These granules are much smaller than cross-sections through rod-shaped and filamentous mitochondria, or sections through mature secretory granules. Their size, form, and location strongly suggest that they are the early stages of secretory granules. Unfortunately, no stages intermediate between these granules and the mature granules of secretion were found. During the stage when the detached argentophilic material is present in the supranuclear region (1–2 hours after feeding), it would appear that the total number of mitochondria is considerably greater than in the cells of fasting animals. It is unfortunately difficult to determine whether there is a real increase in number or whether many of the mitochondria are hidden by secretory granules, and, therefore, invisible until there is a considerable decrease in the number of granules. No evidence suggesting the division of the mitochondria or their direct participation in secretion was observed.

Mucous Neck Cells

These are large columnar cells, cylindrical in shape, resembling to some extent goblet intestinal cells. The supranuclear pole is filled with mucus formed into a goblet, but without distending the cell as in the case of the intestinal goblet cells. The Golgi material, easily demonstrated by both silver and osmic methods, forms a cup-like structure adjacent to the lower border of the accumulated mucus (Text-fig. 1, fig. 11; Pl. I, fig. 15). Mitochondria are present as extremely fine filaments which are shown most clearly in material fixed according to the method of Meves and stained with acid fuchsin. Their number is always very limited and they are present almost exclusively in the subnuclear cytoplasm (Text-fig. 1, figs. 13–14). The secretory cycle and accompanying changes are analogous to those described by other workers for goblet intestinal cells, with the exception that the secretory granules are extremely small and fill all the supranuclear zone. The granules show a marked affinity for acid fuchsin, but not for haematoxylin. These numerous granules gradually accumulate at the glandular pole of the cell where a uniform mass of mucus secretion is formed and extends towards the Golgi zone (Text-fig. 1, fig. 14). The characteristic reaction for mucus is given by the uniform mass, but not by the small granules. During a fast, practically all these cells are filled with secretion. The elimination of mucus starts with the intake of food, and follows a rather peculiar course. The outer masses of secretion are passed into the lumen where they may be easily demonstrated. Elimination is never complete, and the lower part of the mucous mass is immediately replaced with newly produced secretion. The Golgi material, during increased secretory activity, expands and increases in volume (Text-fig. 1, fig. 12). With the accumulation of secretion it moves closer to the nucleus and becomes smaller. No noticeable changes in shape, location, or in the number of mitochondria were observed in any of the preparations.

Surface Epithelium

The surface cells, bordering the gastric lumen, are of the regular, simple columnar type. The nucleus is oval and lies in the middle of the cell. Numerous granules, spherical in shape, fill the supranuclear region. They vary in number, depending on the functional stage, but are always present during fasting and after feeding (Text-fig. 1, fig. 7). As in the mucous cells, these granules stain more readily with acid fuchsin than with haematoxylin.

The Golgi material is a complicated structure which resembles a network, and lies between the nucleus and the accumulated secretory granules at the glandular pole (Text-fig. 1, fig. 5). The mitochondria, very minute in diameter, and almost exclusively filamentous, are scattered more or less evenly in the supra- and subnuclear cytoplasm. They are few in number, especially in comparison with those of the intestinal epithelium (Text-fig. 1, fig. 7). Immediately after feeding the number of secretory granules decreases markedly, while simultaneously new granules originate in the Golgi zone and are scattered through the supranuclear region (Text-fig. 1, figs. 6–7). Within 2 hours after feeding the accumulation of small granules, close to the distal cellular margin, is as dense as in the fasting condition. Owing to their minute diameter, the mitochondria are very difficult to observe, and no marked changes in their form or disposition were noted.

The neck cells which lie between the mucous neck cells and the zymogenic cells are transitional forms, and in outline resemble the zymogenic cells. They show exactly the same internal cytological structure as the surface epithelium, and the description of the latter applies equally to these transitional cells.

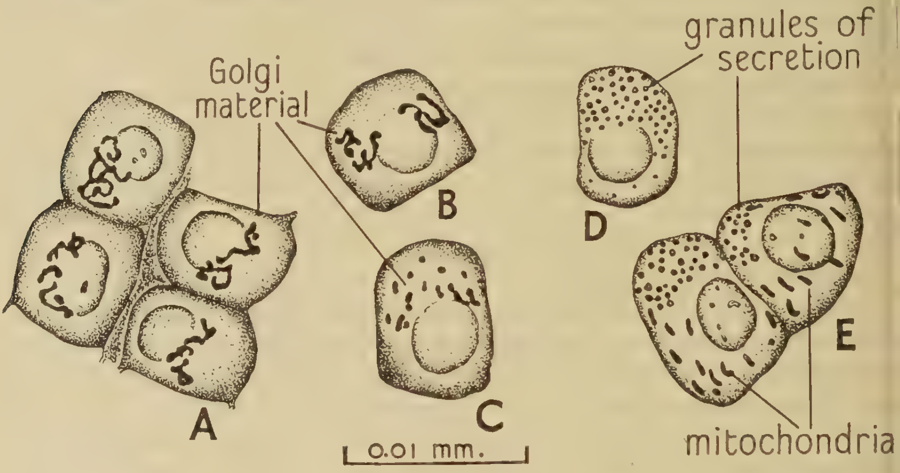
Gizzard

The gizzard is a peculiar structure and in many respects differs markedly from the proventriculus. Its mucous membrane has a thick, horn-like inner lining, which is generally believed to be a secretory product of the epithelium lining the tubular crypts.

The epithelial lining consists of a single layer of cuboidal cells arranged in protruding lamellae which form simple elongated crypts. These are filled with keratinoid material which is connected with the outer horn-like cover. The cuboidal cells near the apices of the lamellae are more elongated, and often club-shaped, with a wider part directed towards the outer keratinoid mass. A very large spherical nucleus generally fills more than half the cell, so that the latter often bulges into the lumen of the crypt. The epithelial cells which line the crypt are strikingly different from other gland cells. Their peculiar behaviour suggests that they belong to a separate group of cells and resemble more closely epithelia undergoing keratinization, first described by Deineka (1912).

Examination showed that only the cells at the bottom of the crypts and the lower parts of the lamellae are normal cells in which all the components are visible. Proceeding from the bottom of the crypt to the apices of the lamellae, the cells show progressive degenerative changes which finally lead to their death. In material fixed by Meves's method and stained with acid fuchsin-picric acid, whole cells embedded in the lower parts of the keratinoid mass are clearly seen. Forty-eight hours' fixation in Meves's fluid is sufficient for the osmium tetroxide slightly to darken the numerous keratinoid granules which fill the cells as well as the intercellular spaces between the dead cells. The cells embedded in the keratinoid mass seem to be preserved by the latter. The keratinoid cover is not a structureless mass, but consists of numerous granules, dead cells, and a more or less uniform cementing mass which stains a deep yellow with picric acid. The more superficial layers, however, are stained a uniform yellow and do not show any structural details.

In the cells of the lower parts of the crypts the Golgi material is a reticulate structure similar to that of the other gland cells. It lies at one pole of the nucleus, or, collar-like, surrounds the middle region of the nucleus (Text-fig. 2, figs. A, B). A few small granules are seen scattered throughout the twisted filaments of the Golgi material. In the cells situated towards the apex of the lamellae, the Golgi material is less voluminous and is progressively broken up into short rods and granules (Text-fig. 2, fig. C). In the club-shaped cells, on the apices of the lamellae, Golgi material is not revealed by any of the methods used. In the nuclei of cells situated towards the apex of the lamellae there is a marked increase in the number of darkly stained granules. In the vicinity of the top of the lamellae the nuclei appear to shrink, and the nucleoplasm is uniformly darkly stained.



Text-fig. 2

Figs. A and B. Cells from the bottom of the crypt, with Golgi material.
Fig. C. Cell near the top of lamellae, with Golgi material breaking up.
Fig. D. Cell near the top of lamellae, with granules of secretion.
Fig. E. Cells with mitochondria and granules of secretion.

Rod-like mitochondria, oriented parallel to the long axis of the cell, are present in cells in which the Golgi material is well developed; as a rule they are less numerous than in the other gland cells (Text-fig. 2, fig. E). In cells towards the apex, where the Golgi material is breaking up, the mitochondria are less numerous and finally disappear.

Secretory granules are few in number in the basal cells and are present in the supranuclear zone. They gradually increase in number in the cells situated nearer the top of the lamellae (Text-fig. 2, fig. D). In the cells in the vicinity of the apex of the lamellae, in which the Golgi material is breaking up, the granules reduce osmium tetroxide; consequently they assume a yellow-brown colour like the surrounding keratinoid mass.

Fasting and feeding do not induce cytological changes in any of the cells of the gizzard. The formation of the granules seems to be progressive and continuous. The few granules present in the cells at the bottom of the crypt, and the relatively small amount of Golgi material which does not show any changes during the phases investigated, indicate that the formation of the secretion is very slow and is not influenced by the process of digestion. Expulsion of the secretory granules must take place more slowly than production, so that the number of granules gradually increases within the cell. The process of secretion appears to slow down in the older cells at the top of the lamellae and finally leads to their exhaustion and death. These cells do not disintegrate, but become embedded in the secretory product.

Fragmentation and terminal disappearance of the Golgi material and of the mitochondria are no doubt signs of the degenerative processes taking place within the cell. The few mitotic figures seen in some of the material at the bottom of the crypts may indicate the method of replacement of old cells. As elimination of the old cells is slow, cell division resulting in new cells is of infrequent occurrence.

The slow secretion of the epithelial cells, independent of digestion, indicates that their function is to renew the keratinoid cover worn away by the mechanical function of the gizzard.

Intestinal Epithelium

As regards histological classification and description of the cells of the intestinal epithelium, there is practically nothing to add to the detailed works of Clara (1926–7). The cells which compose the intestinal lining are arranged in a single columnar layer. In addition to the main epithelial cells (with epithelial cells of the Lieberkühn crypts as their variant) and goblet mucous cells, two kinds of cells enter into the composition of the intestinal lining; these are the chromaffine and Paneth cells.

The main epithelial cells are referred to in this work as epithelial cells, and are the most numerous type. The goblet cells, scattered between the epithelial cells, gradually increase in number from the duodenum to the rectum, where they are nearly always predominant and in some cases may form the entire intestinal lining. The number of goblet cells present in a particular region depends on the physiological phase; they increase during a fast and during the later stages of digestion, but diminish considerably in the first hours after the intake of food. The goblet cells are numerous in the crypts and diminish in number towards the top of the intestinal villi.

Epithelial Cells

These cells vary in shape and size, depending upon their situation and the degree of contraction of the intestinal villi. The oval nucleus is situated in the basal half of the cell, but is usually closer to the middle of the cell than to the basal pole. The outer pole of each cell has a characteristic structure known as the striated border. It is a well-marked, girdle-like extension of uniform texture with delicate longitudinal striations; this region is free of cytoplasmic components. The cell membrane is very faintly marked.

The epithelial cells of the Lieberkühn crypts differ from those of the villi. The nucleus is large and is situated close to the basal membrane. A striated border is absent, and the cytoplasm has a much stronger affinity for dyes, such as haematoxylin and acid fuchsin, than that of the cells in the villi. This property creates some difficulties in cytological work, and demands careful handling during staining and differentiation.

In addition to the typical epithelial forms, a number of narrow, darkly stained cells are visible. Clara (1926) suggested that these are normal epithelial cells compressed by neighbouring goblet cells, or that they are goblet cells subsequent to the discharge of their mucous content. During the present investigation, it was noted that these cells become more numerous soon after feeding, while the goblet cells diminish considerably in number, and that they are very seldom present between the goblet cells. This strongly supports Clara's second suggestion that the dark cells are regenerating goblet cells. Mitotic divisions are always present, but are found almost exclusively in the crypts.

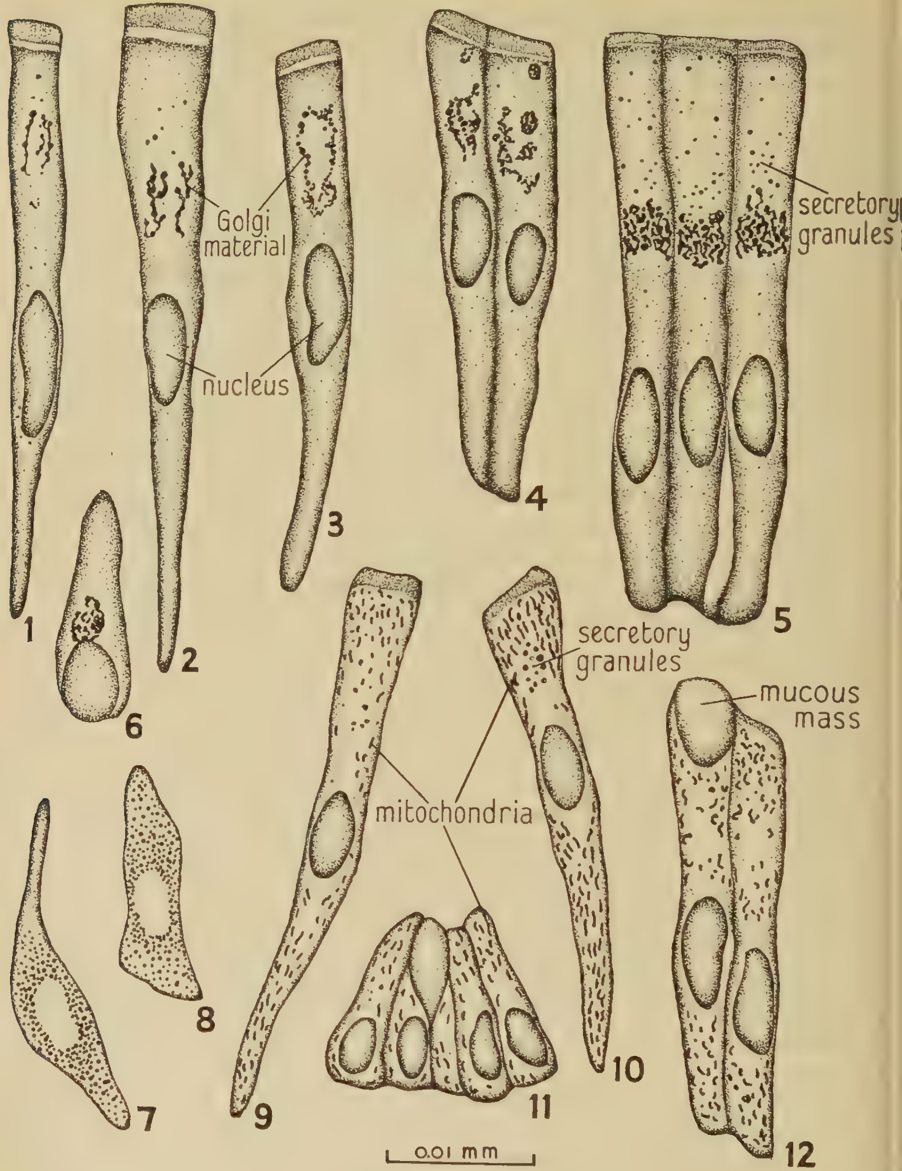
Little need be said regarding the morphology of the resting cells of specimens killed after 24 hours' fast, except that the basal half of the cell appears to be more conically compressed than during digestion. In sections stained for mitochondria, no granules other than granular mitochondria, or cross-sections of rods and filaments, are present. After feeding the cell appears to be more uniform in width and the nucleus seems to move slightly from its central position towards the basal third of the cell. A light area without any distinct border becomes visible in the supranuclear region, and increases in sharpness and size as digestion proceeds. It corresponds closely with the area occupied by the Golgi material and is the negative image of the latter. In the rows of cells on a villus the areas form a lighter zone above, and parallel to, the anterior limit of the nuclei. Secretory granules, scattered in and above the Golgi field, are visible and increase in number during the phases of secretory activity (Text-fig. 3, figs. 9–10). There appears to be no fundamental difference in the behaviour of the cells of the Lieberkühn crypts. Here also secretory granules appear in the Golgi field.

Golgi material. After feeding, the response of the Golgi material was found to vary greatly at different levels of the intestinal canal. The best results were obtained by the examination of sections of the canal immediately above and below the line marked by the descending bowel content. The duodenum and the upper part of the ileum gave the quickest and most marked response, and are, therefore, the most favourable regions for observations.

After a 24 hours' fast the Golgi material in the epithelial cells throughout the entire length of the intestine shows approximately the same morphological pattern. The Golgi material occupies a median position between the nucleus and the lumen; in this phase it is very small and consists of a few thin rods and threads with granular swellings of variable size, usually not more than twice the thickness of the threads themselves. The rods and threads are arranged more or less parallel to the longitudinal axis of the cell. A few cross-links, usually of very small diameter, connect the longitudinal threads so that the Golgi material appears as a simple elongated reticular structure (Text-fig. 3, figs. 1–2; Pl. II, fig. 13). In this phase, the Golgi material is most difficult to impregnate successfully. The Mann-Kopsch method gave the most satisfactory results, but the tissue must be kept for over 3 weeks in 2 per cent. osmium tetroxide at room temperature. Prolonged osmication is needed for the other osmic methods. Silver impregnation may be carried out for the usual length of time, but unfortunately, owing to the many silver deposits, may be used only as a control.

After feeding, parallel with the movement of the food contents inside the bowel wall, marked changes in the Golgi material quickly take place. At the time when food material reaches the duodenum numerous secretory granules, arranged in compact clusters and longitudinal rows, appear in the Golgi region. The clusters create the illusion that fragmentation of the Golgi material takes place (Text-fig. 3, figs. 3–4; Pl. II, fig. 14). This phase is of short duration and mature granules soon begin to move away from the Golgi field. At the same time the Golgi material begins to increase markedly in size and its reticulate structure becomes more evident. It is now a complicated structure with numerous granules situated along the connecting links and between the meshes of the network (Text-fig. 3, fig. 5; Pl. II, fig. 15). Many granules which have moved away from the Golgi zone are present above it, and the stages of their development and terminal accumulation below the striated border may be followed with ease. The first cells to respond to stimulation are situated on the top of the villi, and the onset of secretion in the cells near the crypts begins later. This facilitates observation of the consecutive phases of secretion, which may be followed in a single villus. Approximately 1 hour after feeding, the Golgi material of the cells of the duodenum is a strong reticular structure stretching between the lateral cell borders. In neighbouring cells it is always at the same level and its position is not influenced by the distance of the nucleus from the basal pole of the cell; consequently, longitudinal sections across the villi show a wide, deeply impregnated belt situated parallel to and above the nuclei. Numerous granules in the supranuclear region give this part of the cell a much darker appearance than the remainder of the cytoplasm (Pl. II, fig. 15).

Analogous changes were easily followed in the proximal parts of the ileum, parallel with the progress of the bowel contents. These changes are less striking towards the terminal part of the small intestine, and in the lower part of the ileum they are scarcely, if at all, noticeable. In the caeca and rectum no changes were observed. The maximum manifestation of the stimulating action of food seems to be about 1 hour after direct contact with the contents



TEXT-FIG. 3.

All figures of intestinal epithelium.

Figs. 1–6 from Mann-Kopsch preparations; 7–12 from Regaud preparations.
Figs. 1 and 2. Cells from villi, showing Golgi material; after 24 hours' fast.
Figs. 3 and 4. Cells of the upper part of intestine upon direct contact with food; fragmentation of Golgi material and new secretory granules.
Fig. 5. Cells of duodenum 1 hour after feeding; Golgi material hypertrophied and secretory granules shown above it.
Fig. 6. Cell of glandular crypt showing Golgi material; after 24 hours' fast.
Figs. 7 and 8. Chromaffin cells.

of the intestine. From that time slow retrogressive changes set in. In specimens which had constant access to food the shape of the Golgi material varies little, and is intermediate between that of resting and active cells of specimens previously starved.

Besides the morphological changes, physico-chemical changes appear to take place in the Golgi material. With the onset of secretory activity there is a marked increase in the power of the Golgi material to reduce osmium tetroxide; therefore a quicker and deeper blackening of the Golgi material is a visible manifestation of these changes. As already pointed out, resting cells, fixed in Mann-Kopsch, must be kept in osmium tetroxide for 3 weeks in order to give satisfactory results. In cells previously brought to the resting phase by fasting and subsequently stimulated by direct contact with food, the Golgi material is blackened after 2 weeks in osmium tetroxide.

In cells prepared by osmic methods very small impregnated granules (mostly on the border of microscopic visibility) are present in various parts of the cytoplasm. In some cells a small agglomeration of granules is visible just below the nucleus. In the nuclear region, where only a narrow strand of cytoplasm lies between the cell membrane and nuclear membrane, the granules seem to lie on the surface of the nucleus and are deeply impregnated. The presence of granules below the nucleus in some cells suggests that their passage from the basal part of the cell towards the outer pole is obstructed by the nucleus. In the Golgi zone they are intermingled with the larger secretory granules. The granules increase in number during the secretory phase; this suggests that they may be the prototypes of secretory granules which at a later stage are intimately connected with the Golgi material. Further investigation on the nature of the granules is desirable.

Mitochondria. In their distribution and arrangement the mitochondria of the intestinal epithelium of the domestic fowl follow the general pattern described by many investigators of vertebrate material. The anterior intestinal cells, where secretory response is most pronounced, were chiefly used for examination. In the resting phase two areas of mitochondrial aggregation were observed—one immediately below the striated border and the second in the basal subnuclear region of the cell. The aggregation at the outer pole of the cell is denser than that in the subnuclear region. In the rest of the cytoplasm the mitochondria are less numerous and are fairly equally distributed; they are practically absent from a small area directly above and below the nucleus (Text-fig. 3, figs. 9–10). All forms of mitochondria are present, but wavy and slightly curved filaments of various lengths predominate. Short rods and granules, though few in number, are also encountered. Short

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Fig. 9. Cell from villus of upper part of intestine after 24 hours' fast; to show mitochondria.

Fig. 10. Cell from villus of the upper part of intestine half an hour after feeding; to show mitochondria and secretory granules in Golgi field.

Fig. 11. Cells of glandular crypt after 24 hours' fast; to show mitochondria.

Fig. 12. Cells of caeca; mitochondria irregular in shape and arrangement.

filaments and rods appear to be more characteristic of the subnuclear zone, where only a few granules are seen. Polar orientation of the mitochondria parallel to the long axis of the cell is already well known and has been described by most workers on the intestinal epithelium.

Soon after the intake of food, at a time which closely corresponds to the first visible secretory response of the Golgi material, the mitochondria undergo certain changes. The more deeply stained segments, and bleb-like swelling which are present during the resting phase, disappear and evenly stained forms predominate. The agglomeration of the mitochondria close to the striated border becomes less marked and a more even distribution is observed in the supranuclear zone. Short and granular forms appear to diminish in number but do not entirely disappear. A characteristic feature of the mitochondria in this transitory phase, which is of shorter duration than the analogous period in the history of the Golgi material, is a decreased affinity for ordinary dyes such as acid fuchsin and haematoxylin. Consequently, more skill and care are needed in differentiation than during the other stages. It seems that Saito, describing his 'primary mitochondria', unwittingly referred to this phase in which the mitochondria are most difficult to stain. Secretory granules appear in the Golgi area which now contains few mitochondria and is visible as a light zone. While the small early granules stain deeply, the older ones, which become rather vesicular, stain very faintly, but are shown most successfully in sections stained with acid fuchsin. Subsequently to this phase, the mitochondria exhibit a greater variety of form; their tortuous lines make them similar to those described for the resting phase.

Towards the posterior part of the alimentary tract, the mitochondria of the epithelial cells show greater variability; the threads are less regular and do not show such a marked polar orientation as in the duodenum. Bleb-like swellings, granular and ring-like forms are more numerous (Text-fig. 3, fig. 12). The response of the mitochondria in the lower parts of the intestine is slower and decreases progressively towards the end of the small intestine. In the cells of the crypts of Lieberkühn, shorter filaments predominate over the other forms, and the mitochondria appear to be less numerous than in the cells of the villi (Text-fig. 3, fig. 11). No change of form or disposition was noted in the mitochondria of these cells.

Goblet Cells

The present account does not provide much new information on the goblet cells, and most of the observations made confirm the descriptions of previous workers on other animals.

The goblet cells of the domestic fowl differ markedly from those of mammals. They are always in the form of a neatly shaped goblet. The lower part, which contains the nucleus and most of the cytoplasm with its components, is narrow. The nuclei of the goblet cells usually stain more deeply than those of epithelial cells. The Golgi material is situated between the nucleus and the lumen and forms a network composed of rods and filaments of variable thickness (Text-fig. 4, figs. 1–6).

In the early phases of secretion the Golgi material is present, for the most part, in the form of short segments and threads (Text-fig. 4, figs. 1-2). With the onset of secretory activity the reticulum becomes much more complicated, forming a cylindrical basket which stretches throughout the whole length of the narrow part of the cell. Secretory granules are visible in intimate connexion with the rods and threads (Text-fig. 4, figs. 3–4). Free osmiophilic granules are seen inside the basket intermingled with non-osmiophilic vesicles. The non-osmiophilic vesicles give a positive reaction with mucus-staining dyes, such as mucicarmine, toluidine blue, and thionine; they migrate from inside the Golgi basket to the outer pole of the cell where they gradually fuse together to form a uniform mass of mucus which replaces the cytoplasm and pushes the cytoplasmic components towards the narrow basal region (Text-fig. 4, figs. 5–6).

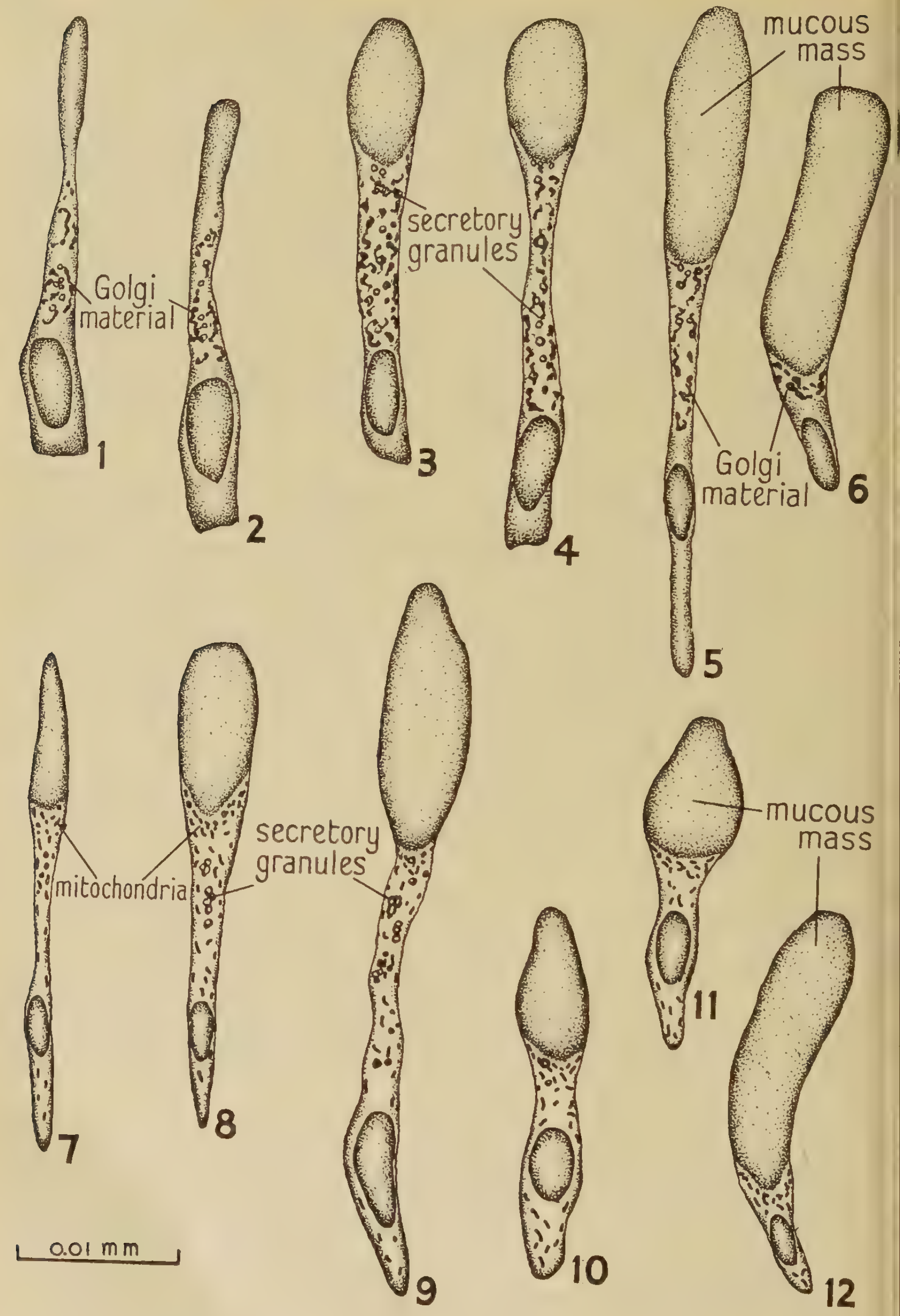
Consecutive phases in the production of mucus may be traced by comparing Golgi preparations with material prepared for the demonstration of mitochondria and counter-stained with mucicarmine. As the formation of the goblet nears completion, the secretory process seems to diminish, and the Golgi material begins to return to its original form. Free secretory granules appear to be short-lived and their transformation into non-osmiophilic mucous vesicles must be rapid. The mitochondria are similar in form to those of the intestinal epithelial cells (Text-fig. 4, figs. 7–12). They are pushed towards the narrow basal part of the cell by the accumulation of the secretory mass in the goblet, and are most numerous close to the membrane separating the cytoplasm from the basal border of the mucous goblet (Text-fig. 4, figs. 11–12). No conclusive evidence was obtained suggesting their direct transformation, or even participation, in the secretory processes.

Chromaffin and Paneth Cells

Only one type of these cells was observed in the material investigated. This type is present throughout the intestine and is most numerous in the crypts. In form, these cells vary from bottle-shaped, with the narrow neck directed towards the intestinal lumen, to spindle-shaped cells lying between the epithelial cells. The large lightly stained vesicular nucleus lies in the central part of the cell (Text-fig. 3, figs. 7–8).

The cytoplasm is filled with a considerable number of granules possessing peculiar properties. In mitochondrial preparations they stain deeply and uniformly with haematoxylin. In impregnated material they blacken uniformly with osmium tetroxide, and as easily as the Golgi material during secretion. During fasting and at all times after feeding the cells behave in the same way and no differences in the number of granules, or in their staining properties, were noticed.

The function of these cells remains obscure, and the present observations do not provide any new information.



TEXT-FIG. 4.

All figures show secretory cycle in intestinal goblet cells.
Figs. 1–6 from Mann-Kopsch preparations; 7–12 from Regaud preparations.

DISCUSSION

Proventriculus

The present account of the gastric epithelia of the fowl supplements previous inadequate cytological studies on the avian stomach, and provides certain information not hitherto recorded.

The present investigation demonstrates that the cytoplasmic components of the different types of gastric cells undergo marked changes during periods of fasting and after feeding. The changes observed vary considerably in the different types of cells; they are most prominent in the zymogenic cells and least striking in the cells of the surface epithelium. In all types of gastric cells the maximum accumulation of secretory material occurs after a 24 hours' fast. Subsequently to feeding there is a gradual decrease in the amount of accumulated secretory material, and the progressive evacuation, which begins immediately after food intake, reaches its peak about 2–3 hours after a meal. After that time there is a gradual increase in the amount of secretion present in the cells. A complete evacuation of secretory material does not take place at any time after feeding. All the gastric cells appear to exhibit constant secretory balance with variations depending on the intake of food and periods of fasting.

As regards the cytoplasmic components it is of interest to note that the response in the gastric cells, due to feeding, is noticeably less marked than in those of the intestinal epithelium. This fact, as well as the constant presence of secretory material in all the gastric cells throughout the phases investigated, suggests that during fasting the secretory processes are greatly retarded, but are not completely suppressed and revive after feeding. This suggestion agrees with the observation of Ma, Lim, and Liu (1927), who, using the much more drastic methods of histamine stimulation on dogs with gastric fistula, never succeeded in exhausting the zymogenic cells to such an extent as to free them completely of secretion. Attempts to trace the early stages of secretion in each group of cells indicates that the granules originate in the Golgi field. Parallel with increased production of granules of secretion a moderate hypertrophy of the Golgi material takes place. In the mucous cells and surface epithelium the secretory granules were easily followed from the Golgi material to the outer glandular pole of the cell, but in the zymogenic cells they could only be traced by comparing the impregnated material with sections stained for the demonstration of mitochondria.

Figs. 1 and 2. Cells in the early stages of secretion; Golgi material small, few secretory granules.

Figs. 3 and 4. Cells in advanced stages of secretion; Golgi material hypertrophied, secretory granules more numerous.

Figs. 5 and 6. Cells in terminal stages of secretion.

Fig. 7. Cell in early stages of secretion, showing mitochondria.

Figs. 8 and 9. Cells in advanced stages of secretion; showing secretory granules and vesicles.

Figs. 10–12. Cells in terminal stages of secretion, showing mitochondria accumulated on the border of the mucous goblet.

The Golgi material of gastric cells was investigated by Golgi (1909), who pointed out that it varies greatly in different types of gastric cells. Golgi also directed his attention, and that of many subsequent workers, to the strange location of the Golgi material in the zymogenic cells. D'Agata (1910) attempted to prove that simple traumatic lesions may cause a reversal of polarity of the Golgi material of the superficial gastric epithelium. Giroud (1928), on the other hand, claimed that the equatorial situation of the Golgi material in zymogenic cells is a purely mechanical occurrence due to pressure from the secretory granules. It would seem from the available literature that most of the discussions on the Golgi material in the zymogenic cells and its peculiar topography are based on the original works of Golgi, and on those of Kolster (1913). None of the later workers, except Kopsch (1926) and Hibbard (1942), gave any information on their methods and degree of success in demonstrating the Golgi material of these cells. The present observations agree with the statements of other authors that the behaviour of the Golgi material of zymogenic cells is completely different from that of other glandular cells. The writer, however, believes that previous statements that the Golgi material of the zymogenic cells always lies lateral to the nucleus are inaccurate. A considerable variation in the position of the nucleus in relation to the Golgi material was observed during the present work. At the time when there is a marked accumulation of secretory granules, the nucleus moves towards the basal membrane, and is encircled by the Golgi material in the form of a vertical collar. When the granules decrease in number, the nucleus moves towards the central part of the cell and leaves the Golgi material behind in its former position. The question of the reversed polarity of the Golgi material was a problem which involved much discussion and controversy. The stable position of the Golgi material observed in the present work suggests that in the case of these cells, and perhaps also in other cells, its polarity is purely relative, depending upon the free movement of the nucleus which appears to be independent of the Golgi material. The present observation strongly supports Pollister's conclusion (1938) that the peculiar orientation of the Golgi material in the zymogenic gastric cells is not associated with the nucleus, but is in relation to the zone of discharge of secretion. Pollister stresses further that careful study of the course of the capillaries at the base of these cells makes it clear that the Golgi material is oriented in relation to the course of cytoplasmic flow between the blood capillaries and the secretory surface.

Much work has been carried out on the mitochondria of the zymogenic cells. Perhaps because of difficulties, and in most cases because of failure, to demonstrate the Golgi material the attention of many workers, especially those who looked on the mitochondria as centres of secretion, has been directed to the appearance of these cytoplasmic components in different functional phases. Some of the earlier workers stated that mitochondria are less abundant in loaded zymogenic cells than in those from which the granules of secretion have been discharged (Eklöf, 1914; Tschassownikow, 1927).

The Chinese school of cytologists, following Cowdry's conception, published a series of papers on zymogenic cells in which, with the help of elaborate descriptions, they ascribe to the mitochondria an exclusive, or at least a major, role in secretion (Lim and Ma, 1927; Ma, Lim, and Liu, 1927; Ma, 1928; Ling, Liu, and Lim, 1928). The present observations do not furnish any evidence that the mitochondria take a direct part in cellular secretion. The only changes observed in the mitochondria were restricted to the zymogenic cells, and these were changes of shape and disposition. In the cells of the surface epithelia mitochondria are few in number, and many secretory granules are produced soon after the intake of food; it is unlikely, therefore, that morphological changes of the mitochondria were overlooked.

Intestinal Epithelium

The ease with which the intestinal epithelium may be stimulated to secretory activity has attracted many workers, and extensive observations have been carried out on active and inactive cells of invertebrate and vertebrate animals. Many of the earlier workers noted that the Golgi material and the mitochondria undergo morphological changes which are correlated with functional activity of the cells. Eklöf (1914) claims that he observed a large number of mitochondria which showed greater structural variations during the digestive process than after a fast of 24 hours. Miller (1922) maintains that in the final stage of secretion in rats mitochondria are absent from the intestinal epithelium, while Saito (1933) describes, in the same animals, a breaking up of filamentous forms into granules half an hour after feeding. Recently, Williams (1943) worked on the mitochondria of the intestinal cells of the Japanese salamander (*Triturus pyrrogaster*), and states that, during digestion and absorption as well as during fasting and inanition, all forms of mitochondria are present. He maintains that the correlation between the mitochondria and the stages of digestion is not merely one of shape, but of the relative number of the different forms present and of the distribution of the mitochondria in the cytoplasm. Accounts of the Golgi material of the intestinal epithelia are more numerous and more detailed. Corti (1926) and Liu (1930) are the only authors who claim to have observed the fragmentation of the Golgi material after feeding. Cramer and Ludford (1925), in their study of the role of the Golgi material during fat absorption in mice and rats, observed that the small amount of Golgi material which is present during the fasting condition swells up and enlarges after absorption of fat, so as to form a network which fills the area between the nucleus and the free border. They maintain that the absorption of food material, other than fat, is not associated with any changes in the morphology of the Golgi material. Weiner (1928), using various animals (frog, white mouse, field mouse, white rat, and axolotl), records changes in both the mitochondria and Golgi material during fat absorption. The Golgi material becomes more deeply impregnated with osmium tetroxide, and there is a thickening of the threads of which it is composed. Subramaniam (1938), in his work on the Golgi material in the intestinal cells of *Lumbriconereis*, describes an increase in the number of Golgi grains during secretion, and an intimate relationship between the first secretory granules and the chromophobic region of the Golgi batonnettes. Jacobs (1929) makes a similar claim for the cells of the mid-gut of *Astacus leptodactylus*.

The present account shows that, apart from the morphological and structural differences characterizing a particular group of cells, there are marked differences in the manner of reaction of cells from different regions of the alimentary tract during fasting and feeding. The secretory cycle of the main cells is easily influenced and brought to the resting phase by a short fast; in the mucous goblet cells, however, secretory activity seems to be governed by entirely different factors. Besides this difference, the same kind of cells in various parts of the intestinal tract respond differently, and very probably perform different functions.

Gresson (1934), working on the mid-gut of *Periplaneta orientalis*, described two kinds of cells, i.e. secretory and absorptive; the former, which are filled with secretory granules, are predominant in the upper part of the mid-gut and are very seldom observed in the posterior part. This author maintains that undoubtedly the function of the posterior part of the mid-gut is mainly absorptive. Williams (1943), working on Japanese salamanders, noted that the response of the mitochondria to fasting and feeding is most pronounced in the anterior intestinal cells. During the present investigations it was observed that this is also true of the Golgi material, and that moving from the duodenum towards the posterior part of the alimentary tract the intensity of the changes of the Golgi material diminishes gradually and disappears completely in the lower parts (caecum, rectum). It was also noted that the main epithelial cells when brought to a resting phase by fasting become strongly sensitized to the subsequent administration of food and show prominent morphological changes. The present observations, following the routine of taking samples from the parts above and below the line marked by the descending bowel contents, show clearly that the first apparent secretory changes are initiated by the direct contact of the cell with food. That the cells present on the top of the villi respond more quickly than those situated below supports this statement. The gradual decline and total disappearance of the morphological changes in the posterior part of the intestine are no doubt connected with the various functions of the different parts of the intestinal lining. The present account agrees with previous contributions in that the proximal part of the intestine is chiefly concerned with secretion, and the distal part with absorption. These claims are supported by the following: Striking changes in the Golgi material of the upper part of the intestinal epithelium are visible as soon as the bowel contents reach this region, usually in less than half an hour after the intake of food. It is difficult to imagine that in such a short time digestion could take place to such an extent as to convert the food into an assimilable form, and consequently that the changes in the Golgi are concerned with absorption. Secondly, the progressive phases in the production of the secretory granules clearly indicate that all these granules make their way towards the striated border of the cell. There is no indication that any of the granules take a different course, i.e. towards the basal part of the cell, as they would do if composed of absorbed material. Examination of material from birds killed at various times after fasting and feeding shows clearly that the Golgi material plays a specific part in secretion and that to deny its participation in secretory phenomena would be more than illogical.

Sudden and short-lived changes in the mitochondria during the first transitory phase, when the cell components seem to be mobilized for the secretory process, are an indication of their share in the synthesizing phenomena which terminate in the production of free granules.

The diminished morphological changes of all cell components in the lower parts of the intestinal tract undoubtedly indicate that secretion in this part (except mucus) must be very limited, or even non-existing, and that absorption does not actively influence the cell components.

I wish to express my thanks to Professor James Ritchie for granting me facilities to carry out this work, and to Dr. R. A. R. Gresson for help and valuable suggestions. My thanks are also due to Dr. W. A. Greenwood for kindly providing me with the birds for this work.

SUMMARY

1. *Proventriculus*

The Golgi material of all the gastric cells, except the zymogenic cells, is situated between the nucleus and the lumen of the gland. In the zymogenic cells it always lies at the level of the bottom of the intercellular clefts. The apparent reversal of the polarity of the Golgi material of the zymogenic cells is due to the movement of the nucleus.

The mitochondria of the surface epithelium and the mucous neck cells are very delicate filaments. In the zymogenic cells thick rods and granules are usual. The functional stage is characterized by the presence of long rods with marked polar orientation.

Secretory granules arise in close association with the Golgi material of all the gastric cells. Feeding accelerates the evacuation of secretion and immediately stimulates new production. A total expulsion of secretory granules never takes place in any of the gastric cells.

2. *Gizzard*

The keratinoid material is a secretory product. The Golgi material does not undergo any changes after feeding. The secretory process is very slow and is independent of digestion. Secretion terminates with the degeneration and ultimate death of the cell.

3. *Intestinal epithelium*

The Golgi material of the epithelial cells lies above the nucleus. It shows marked changes of morphological and physico-chemical nature as soon as the resting cell is stimulated by direct contact with food.

The mitochondria are in the form of filaments, rods, and granules. Their polar arrangement is a constant feature. When the cell is first stimulated by food, the mitochondria stain very faintly.

Secretory granules arise in close association with the Golgi material; they move towards the glandular pole of the cell. Morphological changes of the Golgi material and mitochondria diminish and finally disappear in the posterior parts of the alimentary tract.

4. *Goblet cells*

The Golgi material lies above the nucleus. During secretory activity it enlarges greatly; it decreases in mass during the final stage of secretion.

The mitochondria are in the form of filaments, rods, and granules. With the accumulation of secretory material, the mitochondria collect on the border of the mucous mass and in the cytoplasm of the narrow part of the cell.

Secretory granules arise in close association with the Golgi material. The secretory process of these cells is autonomous and is not directly correlated with digestion.

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Description of Plate I (Photomicrographs × 1,200)

Fig. 15. Mucous neck cells; Golgi material in form of basket-like structure below the mucous mass.

Fig. 16. Zymogenic cells, showing Golgi material; transverse section of glandular tubules.

Description of Plate II (Photomicrographs × 1,200)

Fig. 13. Cells from a villus showing Golgi material; after 24 hours' fast.

Fig. 14. Cells from a villus upon direct contact with food; fragmentation of Golgi material.

Fig. 15. Cells from a villus 1 hour after feeding; Golgi material hypertrophied.

The Cytology of the Neurones of *Helix aspersa*

BY

OWEN LEWIS THOMAS, M.D. (N.Z.)

Beit Memorial Medical Research Fellow

(From the Department of Zoology and Comparative Anatomy, Oxford)

With seven Text-figures and one Plate

WITHIN recent years the important investigations of Parat (1928), Hirsch (1939), Worley (1943), and Baker (1944) have extended our knowledge of the form, function, and cyclical activity of the intracellular organoids, more especially with regard to the so-called Golgi apparatus.

No longer is it permissible for the cytologist to rely solely on the appearance produced in cells by the classical methods of silver and osmium impregnation for his identification of these organoids. Such studies must be carefully controlled wherever possible by observation of living cells and by the application of vital and supravital staining. Worley (1946) in concluding his recent review of the Golgi apparatus emphasizes this point of view when he states: 'Studies of the Golgi apparatus should always involve a comparison between fixed and living tissue. No structure in the cytoplasm of either the vitally stained or the fixed cell should be accepted unless there is a fairly perfect correspondence between the two types of material. In general structures that blacken with prolonged osmication, and stain vitally with methylene blue may be considered a part of the total Golgi complement of the cell.'

In this study it was decided to carry out an investigation on living or freshly teased nerve-cells; first by direct observation with transmitted, dark field, and phase-contrast microscopy to identify as far as possible all the intracellular structures which could be identified on the basis of a specific and constantly recurring morphology; secondly to submit these structures to a series of procedures with vital dyes, histochemical tests, and further to record the effects of fixation, staining, and metallic impregnation of these bodies. Throughout the earlier part of this investigation no reference was made to the relevant literature with its host of conflicting views, since it was thought that it would have a baneful influence in forming preconceived and possibly ill-founded ideas of these structures. For this reason the familiar terms, Golgi apparatus, Golgi complex, mitochondria, &c., are not used within the section of this paper describing the observations but are reserved for the subsequent discussion.

After some thought the neurones of *Helix aspersa* were chosen for this study since this mollusc or closely allied species have almost a world-wide distribution and the experiments described in this paper can be reproduced and verified easily by other investigators.

METHODS

The freshly teased nerve-cells of the post- and mesocerebrum of *Helix aspersa* were examined in a few drops of 0·7 per cent. sodium chloride containing 0·2 per cent. of 10 per cent. anhydrous calcium chloride. Transmitted, dark field, and phase-contract illumination were employed in this study. The phase-contract microscope and equipment were identical with that used in a previous investigation of these cells (Thomas, 1947).

Supravital and intravital staining were carried out with neutral red chloride, methylene blue B.D.H. 561213741013I, nile blue, and Janus green B (Höchst) dissolved in the required concentrations in 0·7 per cent. sodium chloride solution containing 0·2 per cent. of 10 per cent. calcium chloride. For intravital staining about $\frac{1}{2}$ c.c. of the solution was injected into the haemocoele with a fine hypodermic needle through the expanded foot of the snail at a point about midway between the head and posterior part of the metapodium. The animals were killed by quick decapitation while in the expanded condition.

For fixed material the following techniques were employed:

1. The acid haematein test of Baker (1946) followed by the pyridine extraction control test.
2. Altmann's acid fuchsine picric acid stain for mitochondria, following Schridde's fluid or Bensley's acetic, osmium, dichromate fixative.
3. The Azan stain of Heidenhain and Masson's technique following Zenker–Formol and Carnoy fixation.
4. Mann–Kopsch technique and the variant described on p. 455.
5. The sudan black B technique of Baker (1944) for the demonstration of the Golgi apparatus in frozen sections.

OBSERVATIONS

The Living Cell

The cerebral ganglia of *Helix aspersa* consist of two lateral masses connected by a supra-oesophageal commissure. Each lateral mass contains a fusion of three separate ganglia—a procerebrum consisting of small cells which according to Hanström (1928) are mainly association neurones, and the mesocerebrum and post-cerebrum. The mesocerebrum contains numerous large cells whose axons after a short course branch diversely. Some of these branches run via the central commissure to the ganglia of the opposite side, whereas the remaining fibres either pass to the pedal commissure or anastamose with the ipsilateral neurones of the post- and procerebrum. The post-cerebrum similarly contains large unipolar neurones which are concerned with tentacle reflexes.

These ganglia can be easily isolated by gentle teasing of the fused mass. Usually the cells of the post- or mesocerebrum were selected for study, as their average size of about 50 μ in diameter makes them very suitable objects for high-power study with the microscope. Occasionally pieces of the small-celled procerebrum may by accident be included in the teased preparation, but with experience in manipulating the material suitable groups of medium-sized cells can be quickly selected and spread out between coverglass and slide.

Particular attention has been paid to the examination of the cytoplasm of these cells with both transmitted and phase-contrast illumination. In addition to the neurofibrils and the very small particles (the microneurosomes) previously described (Thomas, 1947), two further categories (Text-figs. 1 and 2) of intracellular structures could be clearly discerned within these neurones:

1. Elliptical corpuscles with attached granules, hereafter described as spheroid complexes.
2. Minute filaments and coccoid chains.

The Spheroid Complexes

These structures or systems appear as a varying number of small refractile globules or spheroids scattered throughout the cytoplasm, each consisting of a spheroid of optically clear homogeneous material to which under optimal conditions of observation one or more still smaller dark granules can be seen to be attached. These dark granules occur irregularly around the periphery of the spheroid and are best seen by slightly reducing the illuminating pencil of light or by using phase-contrast microscopy.

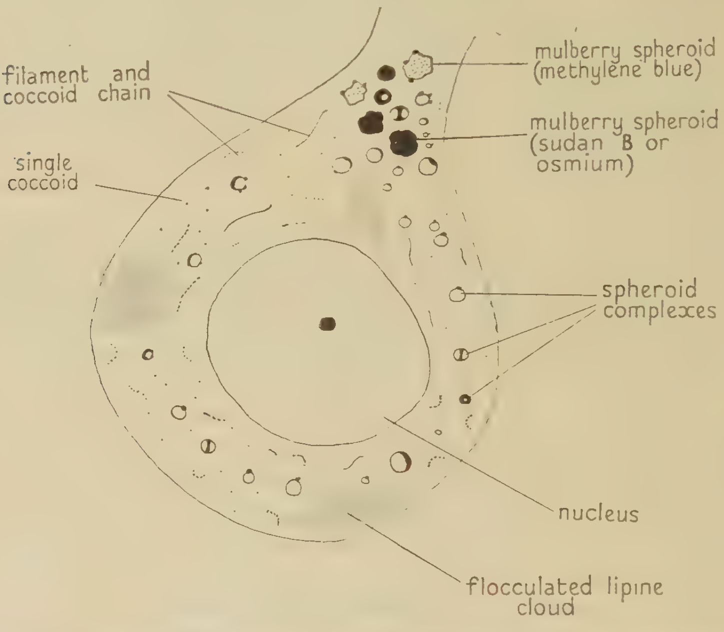
When the cell is crushed between coverglass and slide so as to rupture the cell membrane these structures can be seen to flow out freely with the cytoplasm. On one occasion under these conditions the separation of one of the dark granules from its spheroid was witnessed and the impression was gained that the granule had a morphological identity of its own. It was henceforth decided to give the name spheroid complex to the combination of the colourless spheroid and its accompanying granule or granules. It must be mentioned that the term spheroid complex at this stage is provisional and does not mean that the attached granules or granule can always be demonstrated, since in some cases they may be situated on the deep aspect of the spheroid and (being turned away from the view of the observer) will therefore be invisible.

On the average the spheroids are about 1·5 μ in diameter, but they vary quite considerably in size within any one cell (Text-fig. 1). The attached granules are too small for accurate measurement, but they are considerably larger than the individual microneurosomes. In general these complexes tend to be aggregated in the cytoplasm surrounding the nucleus. Occasionally in the larger nerve-cells large spheroid complexes occur which present a somewhat scalloped outline, resembling a diminutive mulberry (Text-figs. 1, 2, 5).

These mulberry forms are sometimes present in very great numbers within the neurones of fully grown specimens of *Helix* and tend to be accumulated at one pole of the cell. Such a mass may be clearly visible even with the low powers of the microscope, since each mulberry spheroid being tinged somewhat yellow gives to the whole group a distinct golden-yellow colour.

The Filaments and Coccoid Chains

In addition to the spheroid complexes a number of slender colourless filaments, usually somewhat slightly bent towards one end, are scattered throughout the cytoplasm. Further, there may occur tiny chains of 5 or 6 small refractile bead-like structures, resembling somewhat in appearance and size a chain of streptococci (Text-fig. 1). It is characteristic of both these filaments and chains that they are scattered about in a haphazard fashion and are never collected into regular rows like the neurofibrils. A number of discrete single coccoids of the same order of size as the coccoids of the chain forms described above can also be seen within the cell and, in fact, predominate if the filaments or chains are absent.


filament and coccoid chain
single coccoid
mulberry spheroid (methylene blue)
mulberry spheroid (sudan B or osmium)
spheroid complexes
nucleus
flocculated lipine cloud

TEXT-FIG. 1. A composite diagrammatic drawing of a neurone of *Helix aspersa* showing the morphological characteristics of the two categories of intracellular organoids observed with the techniques used in this study.

Vitally Stained Material

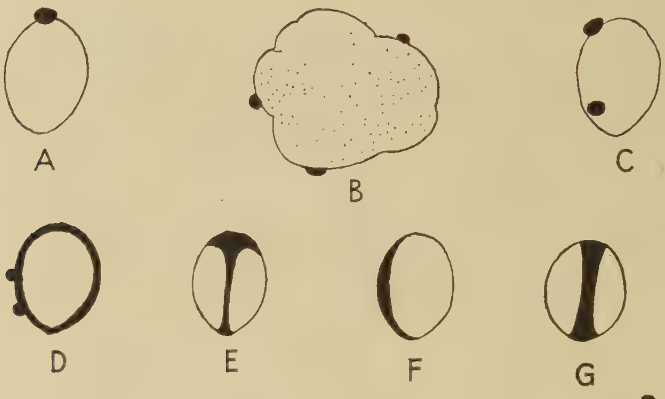
Neutral red chloride. This dye has the power of staining strongly the spheroid complexes to the exclusion of all other structures within the cell. Concentrations of dye as low as 1:100,000 were used supravitally, and although at such a dilution no colour could be detected in the surrounding medium, the cells after a period of 20–30 minutes imbibed the dye, concentrating it very selectively within the spheroid complexes, revealing them as bright brick-red structures.

When neutral red in saline is injected into the haemocoele of the snail and the animal killed within 10–15 minutes, the small dark granules of the spheroid complexes are in addition especially well revealed as very strongly stained granules, whereas the related spheroid itself has under the conditions of the experiment only assumed a slightly reddish tinge. Longer applications of the dye by the injection route will produce an exactly similar appearance to that of the supravital method, but the differential coloration of the complex will be lost. Likewise preparations that are showing full and generalized staining of the complex can be made to reproduce the spheroid plus granule appearance by simply waiting for some minutes longer for the dye to begin to be reduced to the leucobase under the relatively anaerobic conditions obtaining beneath the coverglass. As the red colour fades from the spheroid itself the attached granule or granules are beautifully revealed with the utmost distinction, and will remain as much brighter red granules resisting tenaciously further reduction of the contained dye. In other words, during progressive vital coloration the granule always stains before the spheroid, but later, after the whole system is homogeneously coloured red, the granule then becomes invisible and only finally reappears as reduction to the leucobase produces differentiation of the spheroid complex into its constituent parts.

Methylene blue 1:10,000–1:100,000, like neutral red, produces best results when it is injected in saline solution into the haemocoele. Staining is similarly restricted to the spheroid complexes. The attached granules are particularly well shown. When the dye is allowed to act for short periods of 10–30 minutes, most of the spheroids are of a faint pale-blue colour and their attached granules are by comparison very sharply shown as dark blue. If the dye acts for an hour or more the dark staining of the granule is lost, but stained areas of varying pattern appear to be closely applied to the spheroid. A selection of the various shapes these stained structures may take is represented in Text-fig. 2. This drawing was made during observation of a group of living cells and represents the forms more frequently seen. These darkly staining areas appear as complete or local thickenings in what is usually a pale-staining investment to the spheroid. This investment when stained completely and seen in optical section appears as a rim. In other cases there is an appearance of a crescent which is perhaps due to partial staining of this rim or to staining of a locally thickened area of the rim. In still other cases, columns or complete bands appear around the periphery of the spheroid and these again may be due to viewing the locally stained crescentic areas from the side.

It might be assumed that these appearances of a complete or incompletely stained investment were due to a swelling and elaboration of the simple granule. This cannot be definitely established since in a few cases spheroids possessing rims or localized areas of darkly staining material had distinct granules attached to other parts of their circumference (Text-fig. 2).

After prolonged application of the dye, the material of the spheroid itself in most cases appears to be stained homogeneously throughout. Finally, coincidently with death of the cell, the nucleus and cytoplasm become stained.



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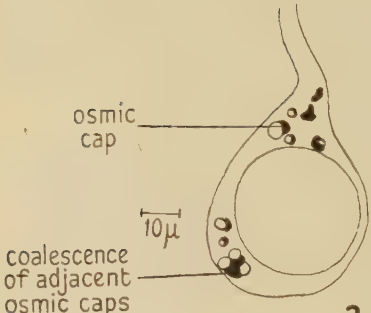

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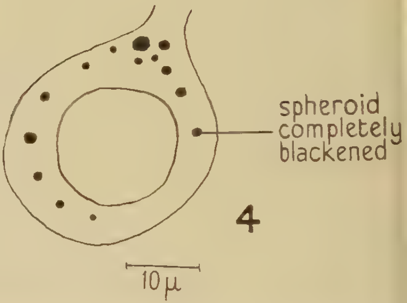


Text-fig. 2. The various shapes of dark-staining granules (A and C), rims (D), crescents (F), and bands (E and G), taken by the chromophil substance when the spheroids are stained with vital methylene blue. B represents a mulberry spheroid with attached granules of chromophil substance. The chromophobe area has accumulated a mass of yellowish Golgi product (the lipochrome of Legendre). With methylene blue this product is but faintly stained, but it is itself coloured with sudan or osmium. Cf. Text-figs. 4 and 5.

Text-fig. 3. A camera lucida drawing of a smeared neurone subjected to warmed osmic vapour. The chromophobe spheroids exhibit exaggerated caps or 'cocked hats'. Coalescence of adjacent caps to form an irregular black lump is illustrated.

Text-fig. 4. A camera lucida drawing of a neurone completely immersed in a solution of osmium tetroxide. The spheroids and mulberry forms are completely blackened. No differentiation into chromophil and chromophobe areas are visible.

Nile blue 1 : 10,000 in saline will give precisely the same results as methylene blue. The attached granules are stained blue while in some cases the substance of the larger spheroids is tinged a reddish colour.

Janus green B (Höchst) 1:10,000 was applied by the supravital method. Considerable difficulty was experienced in getting the stain to work and in successful preparations only a few cells took up the dye. This substance was found to colour the delicate filaments and coccoid chains and individual coccoids, and to leave the spheroid complexes unstained. However, when once staining has occurred the great specificity of this dye is dramatically exhibited. Best results were obtained by compressing a group of cells and then examining those at some distance from the edge of the flattened mass. Cells at the edge usually show diffuse staining of the nucleus and cytoplasm, whereas cells in the centre are wholly unstained. Somewhere along this gradient optimal conditions obtain for the reaction and preparations must be very carefully searched for successful results before being discarded as useless.

Osmium Tetroxide

Freshly teased cells were spread out on a coverglass and then inverted over a cavity slide containing a drop of 2 per cent. osmium tetroxide. Cells at the periphery of the preparation became immersed in the solution, whereas the more centrally placed cells were separated from contact with the osmium tetroxide by a small air space, and hence were only subjected to the action of the vapour. As a result the action of this reagent could be watched progressively under two sets of conditions. In addition some preparations were placed in an incubator at 37° C. and inspected every few minutes in order to observe the effect of heat upon the osmication process.

Cells subjected to the action of the vapour only showed a progressive blackening of the cortex of the spheroid complex. This blackening first appears in optical section as a delicate complete rim, but very soon one portion of the periphery of this rim becomes appreciably thickened to form first a crescent-shaped cap which is finally expanded into a much larger structure resembling in relation to the almost unblackened central core a miniature cocked hat. Text-fig. 3 gives a good representation of these expanded crescents produced by prolonged osmication. Usually the base of the cap or cocked hat of blackened material is continuous with the faint blackened rim to the whole spheroid. The blackened tract of material then resembles a signet ring placed over a ball (the spheroid).

When heat is applied osmicated caps of adjacent spheroids not infrequently are seen to coalesce to form an irregular black lump (Text-fig. 3). The actual process of fusion can, in fact, be witnessed. As coalescence occurs in those cells which were subjected to the heated osmic vapour and not in cells immersed in the cool fluid, it is probable that the heat produces some desiccation of the preparation as a necessary preliminary condition for producing this effect. Coalescence of the osmicated caps of adjacent spheroids was not witnessed in material kept at room temperature.

In cells wholly immersed in a solution of osmium tetroxide the spheroids do not show attached caps or 'cocked hats': instead there is a gradual and progressive blackening of the whole spheroid. At all stages the spheroids appear to be quite homogeneous without differentiation into an outer blackened and inner clear zone as is the case with osmic vapour.

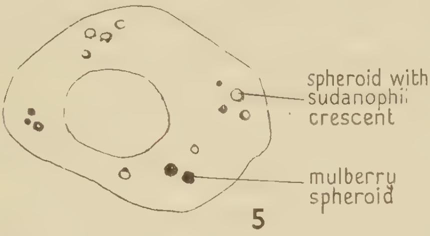
Fixed and Stained Preparations

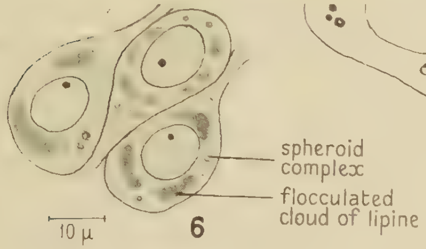
The formal-sudan-black technique of Baker (1944) produces in the cell a picture somewhat intermediate between that of osmic vapour and that of direct immersion in osmium tetroxide solution (Text-fig. 5). Most of the large mulberry spheroids are totally blackened with the sudan, but in addition all degrees of the differentiation of the spheroids into an outer blackened and a pale inner zone occur—signet-ring forms with complete rims and thickened caps, incomplete rims appearing as crescents and sometimes only one or more discrete granules attached to a colourless spheroid. In a large cell the total number of these systems or complexes may be very great indeed, and the casual observer may mistakenly dismiss the smallest complexes as a collection of granules. However, careful study will show that invariably the 'granule' is, in fact, a compound structure. The tiny coccoid rods and filaments stainable with Janus green do not blacken with this technique and remain invisible in these preparations, but sometimes one finds a groundwork of extremely fine black granules forming a dark zone surrounding the nucleus, especially in the larger nerve-cells. These dark areas can only be adequately described as perinuclear clouds, for the precise resolution of their individual particles is a matter of the utmost difficulty. In some cases this material may appear in flocculent, more or less rounded patches occurring throughout the cytoplasm.

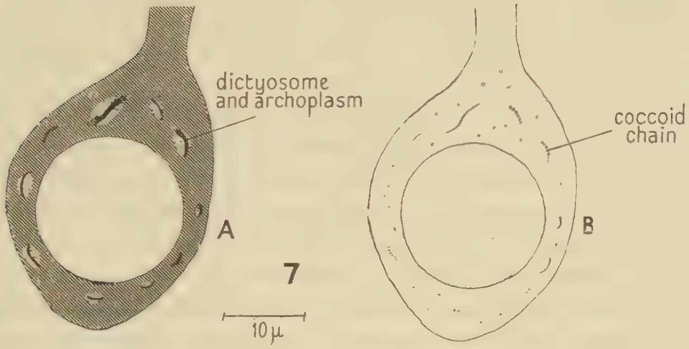
The acid haematein test for lipines after Baker (1946) was applied to this material and the observations controlled by the pyridine extraction test. Baker's technique stains the spheroids a bluish-grey colour. When observed in optical section the impression is gained that the lipines form an outer skin to the spheroid which itself does not give a positive reaction with the acid haematein. In some cells a diffuse cloud of lipine occurs throughout the cytoplasm (Text-fig. 6). In their general morphological distribution these lipine clouds correspond to the diffuse sudanophil material observed with the sudan black technique of Baker (1944). With the pyridine extraction test the cells show a clear cytoplasm with distinct staining nuclei. The spheroid skins and diffuse clouds observed with the acid haematein test are not visible. In some cells complete 'negatives' or 'ghosts' of the spheroids can be seen as clear spaces in the cytoplasm.

With the Mann–Kopsch technique a very different picture of the cell is obtained from that observed in the vital studies and with the techniques described above.

Instead of a number of spheroids with attached rims, crescents, or granules, the cytoplasm is crowded with a large number of osmiophilic curved rods or batonnettes resembling the batonnette of the molluscan 'Nebenkern'. Each rod is surrounded by a more or less osmiophilic zone of cytoplasm. This 'neoformation' within the cell is at first sight very puzzling, and it was thought that these curved rods might be the rims or crescents of the spheroids distorted somewhat by the fixation and thickened by aggregation of osmium particles. Further, it was possible that the spheroid itself might have collapsed and dispersed to form the lighter osmiophilic zone around the batonnettes.







TEXT-FIG. 5. Camera lucida drawing of a neurone prepared by the Baker technique. A close representation of the living and vitally stained cell is obtained. The mulberry spheroids are totally blackened owing to the fatty Golgi product contained within the spheroids colouring with the sudan black.

TEXT-FIG. 6. A group of neurones prepared by the Baker acid haematein test. Flocculated clouds of lipine occur throughout the cell. The spheroids are visible because their skins give a positive reaction for lipine.

TEXT-FIG. 7 A. A neurone prepared by the Mann–Kopsch technique. The so-called Golgi dictyosome and archoplasm can be readily seen, produced by the use of this method. B. The same cell after partial bleaching with the Verrati bleacher. The 'archoplasm' disappears and the dictyosome, greatly reduced in thickness, can usually be resolved into a row of coccoids. This evidence supports the view that the dictyosomes are artifacts produced by the over-impregnation of the mitochondria, and do not represent the true Golgi apparatus of *Helix*.

This idea was put to the test by first locating a suitable group of cells from a Mann–Kopsch preparation, tracing with the camera lucida an accurate outline of the cells, and marking within the exact position of some of the most distinct batonnettes. The coverglass was then soaked off the slide and the preparation passed through xylene and the alcohols to water. The osmium was then completely removed from the sections by immersion in the acid permanganate bleacher of Verrati (see Gatenby, 1939). The slide was then washed in water, placed in 70 per cent. alcohol, and then treated with a saturated solution of sudan black B in 70 per cent. alcohol for 10 minutes. After a quick rinse in 50 per cent. alcohol it was washed in water and mounted in Farrants's medium.

The same cells were then brought into the field of view and a fresh drawing made of the appearances produced by the application of sudan black. By comparing the drawings it was possible to assess the results of the two different techniques upon the same cells.

The 'restained' cells showed very precisely numbers of typical spheroid complexes which were previously invisible in the same cells after the Mann–Kopsch technique. It was obvious that the mercuric osmium fixative had preserved the spheroids but the subsequent osmication had failed to exhibit them. Further, the dehydration, embedding, and bleaching procedures had failed to remove them from the cells. In fact the bleached and sudan-treated sections compared more than favourably with the frozen section material prepared by the original Baker technique.

In this way it was established beyond doubt that the characteristic batonnette obtained by the Mann–Kopsch technique does not bear any positional or morphological relationship to the spheroid complexes exhibited by the Baker technique. Further, as these sudanophil spheroids can be definitely correlated with the spheroids seen in the living cell and the vitally stained preparations, whereas the osmiophilic batonnette did not appear to have such a counterpart, it was thought possible that perhaps the batonnettes were either artifacts produced by non-specific reduction of osmium tetroxide or perhaps a structural component of the cell which had hitherto resisted all attempts to disclose it.

This problem was attacked by arranging for a number of 'successful' Mann–Kopsch preparations exhibiting intracellular batonnettes to be bleached in the Verrati bleacher for graded periods of time so as to reproduce in a series the various appearances of the batonnettes as the osmium was regressively removed. Experimentally this regressive technique is a much easier procedure than to arrange for a series of progressive impregnations of the batonnette.

The results obtained were very illuminating. The first part of the reduced osmium to disappear during the bleaching process is the brownish zone surrounding the batonnette. This is followed by the gradual removal of osmium from the rod itself which consequently becomes much thinner in appearance. Not infrequently some of the batonnettes are reduced to a row of distinct cocci. These stages are illustrated in Text-fig. 7. Finally, if the bleaching is continued all the osmium can be reduced from the sections.

The absence of any impregnation of the spheroids by the Mann–Kopsch technique was somewhat unexpected, as these bodies had already been impregnated successfully by osmic vapour and with immersion in simple 2 per cent. osmium tetroxide. The combination of mercuric chloride with osmium tetroxide in the Mann–Kopsch fixing fluid was considered to be responsible for the shifting of the selectivity of the osmium from the spheroids to the counterparts of batonnettes in the living cell. A series of experiments was planned with variants of the Mann–Kopsch technique, in which the fixative action of the sublimate was dissociated from the subsequent impregnating action of the osmium tetroxide. The following variant proved the most reliable for impregnating the spheroids with osmium:

1. Fix whole ganglia in a saturated solution of mercuric chloride in 0·75 per cent. sodium chloride.
2. Remove excess of mercuric chloride by thorough washing in running water for 12 hours.
3. Leave in distilled water overnight.
4. Transfer to a tube containing enough of a simple 2 per cent. solution of osmium tetroxide to cover the tissue, close the tube with a rubber stopper, and leave in the dark at room temperature for 1–6 days.
5. Pieces should be removed at the end of each day and washed for 3 hours in running water.
6. Dehydrate in alcohols, embed, and cut.

With this method the complete spheroids are thoroughly impregnated and appear as a number of homogeneous black globules.

Differentiation of the spheroid complex into darker rims or caps attached to lighter more translucent spheroid bodies is not evident. If these preparations are now regressively bleached with the Verrati bleacher the impregnation can be shown to extend evenly throughout the whole mass of the spheroid. As the result of bleaching a clear rim appears at the periphery of the black globule and this increases *pari passu* with the reduction in its size until finally a completely decolorized negative or 'ghost' of itself is produced. Such a chain of events can only be consistent with the progressive removal of the outer layers of a homogeneously-blackened spheroid.

Similarly it was found that the spheroids can be impregnated, although rather faintly, during the course of fixation and without subsequent post-osmication if tissues are placed in Schridde or Bensley's acetic osmium dichromate fixative. The blackish spheroids are at once visible on removing the paraffin from the sections and before staining. Some sections exhibiting this phenomenon were stained by the technique of Altmann with acid fuchsine and differentiated in aqueous picric acid.

A number of the lightly osmicated spheroids prepared by this method were seen to possess a clearly differentiated bright fuchsinophil rim which in some cases showed local thickening, giving to the whole a signet-ring appearance exactly comparable with the similar appearances produced by osmic vapour.

The Altmann technique also reveals the presence of slender rods and coccoid chains together with individual cocci. These structures are morphologically identifiable with the filaments and chains exhibited with Janus green B used as an intravital stain on living cells.

The staining reactions to acid fuchsine of the two distinct categories o intracellular bodies, the attached rims or caps of the spheroid complexes and the detached and separate coccoid chains or filaments, thus appear to be identical.

DISCUSSION

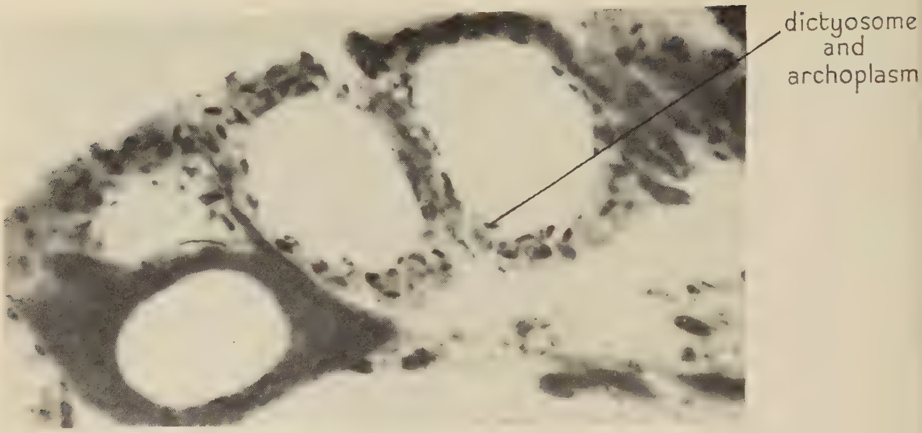
It now remains for us to review the various categories of intracellular structures described above in the light of previous investigations upon the invertebrate nerve-cell, and to endeavour to correlate the particular structures seen within the living neurone with the appearances produced in these cells by application of the technical procedures of fixation, staining, and metallic impregnation. Four distinct and apparently unrelated definitive cellular components have been described by other authors as occurring in these cells:

1. The Golgi apparatus.
2. Mitochondria.
3. Metaplasmic granules (lipochrome of Legendre, neuro-secretion granules of Scharrer, &c.).
4. Nissl substance or tigroid bodies.

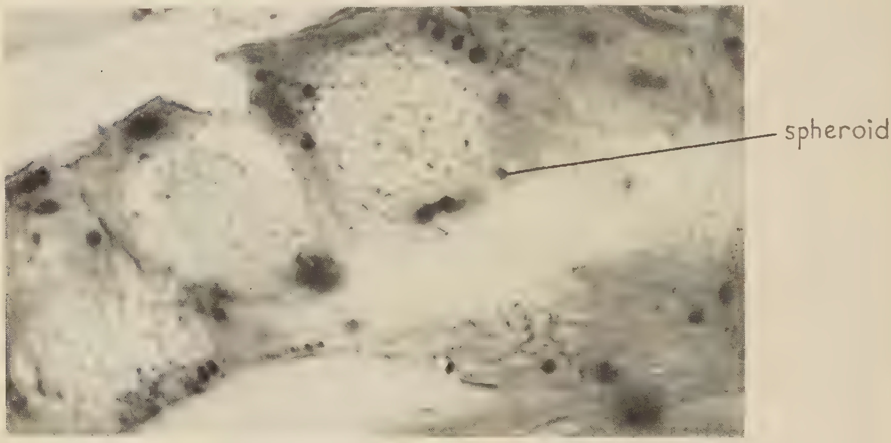
The Golgi Apparatus

A number of workers such as Legendre (1909), Kolatchev (1916), Brambell (1923), Brambell and Gatenby (1923), and Boyle (1937) have described the Golgi body or dictyosome of the molluscan nerve-cell as consisting of a somewhat curved osmiophil batonnette associated with a more or less chromophobe 'archoplasm'. Bodies answering to this description can be readily seen following the Mann–Kopsch technique. In order to prove whether or not these appearances were due to partly impregnated spheroids or perhaps spheroids distorted during fixation, Mann–Kopsch preparations were bleached and recoloured with sudan black. The results of this experiment are illustrated in Plate 1. A photograph (upper figure) was first taken of the dictyosomes, and another after bleaching and restaining the preparation (lower figure). By comparing the two appearances of the same cells it can only be concluded that the spheroids (which can be seen in the living cell) are entirely different bodies from the batonnettes and do not in any way contribute to their production in the fixed cell.

Recently Worley and Worley (1943·4) and Worley (1946) in a series of interesting papers have given very strong support to the view of Hirsch (1939) and others that the true Golgi apparatus consists of a complex composed of chromophil and chromophobe portions. They base this differentiation on the reaction of the apparatus during the life of the cell to a dilute solution of methylene blue. Worley (1946) stressed, however, that the methylene-blue-staining bodies must also blacken with prolonged osmication before conclusive proof of their being part of the total Golgi complement of the cell



Photomicrograph of a Mann–Kopsch preparation showing a typical rod-like dictyosome embedded in a mass of archoplasm.



The same preparation following bleaching with the Verrati bleacher and recoloured with the Baker sudan black technique for the Golgi apparatus. The identical cells are rephotographed. The dictyosomes and archoplasm are lost or the dictyosomes are reduced to fine mitochondria-like threads or coccoid chains. A spheroid complex showing differentiation into chromophil part and chromophobe centre is shown. It should be noticed that this complex remains invisible in the Mann–Kopsch method.

THOMAS.—PLATE I

is obtained. Baker (1944) gives good reasons for believing that the methods used to produce the classical Golgi network cannot be relied upon to give an accurate picture of the structure of the Golgi element during life. Using sudan black B, a colouring agent with an intense affinity for lipoids, he has developed a new technique for showing the Golgi element which has given valuable results on a number of diverse cells, including the cells of the anterior mesenteric ganglion of the rabbit. In these vertebrate nerve-cells Baker describes the Golgi apparatus as consisting of a number of separate vacuoles each surrounded by 'an intensely sudanophil skin which is not uniformly thick all over'. In optical section this skin appears as a ring or crescent enclosing a colourless centre or vacuole. These compound bodies closely agree with the chromophil and chromophobe complexes of Worley and Hirsch.

Young (1932) in cephalopod material observed numerous neutral-red-staining spheres, with attached methylene-blue-staining granules scattered throughout the cytoplasm of the nerve-cells. He believed that similar methylene-blue-staining and osmiophil granules in close proximity to the dispersed spheres probably represented the Golgi apparatus. He could find no evidence of either a Golgi batonnette or a classical network within his material.

Covell and Scott (1928) and Murray and Stout (1947) working on the vertebrate nerve-cell found a close correlation between the neutral-red-staining and osmiophil granules and identified these bodies as a dispersed Golgi apparatus.

Ever since the pioneering work of Parat (1928) we have been entering a new period of cytological inquiry wherein the old criteria for the identification of the Golgi apparatus based on the use of silver and osmium impregnation techniques without adequate control by vital studies must be seriously questioned.

The earlier work on the molluscan 'Nebenkern' and the questionable identification of its contained rod-shaped and easily visible lepidosomes as the Golgi body of the spermatocyte has undoubtedly had its effect upon all subsequent work on invertebrate nerve-cell cytology. Workers have only used those variations of metallic impregnation techniques that successfully produce in the cell a lepidosome-like structure. Slides which show an impregnated curved rod or batonnette have been hailed as 'good' preparations of the Golgi apparatus, and slides not showing this appearance have been arbitrarily rejected as failures. From my own observations I have come to reject the hypothesis that the batonnette and its attached 'archoplasm' represents any part of the Golgi body of *Helix* neurones.

On the contrary, the invertebrate nerve-cell has been shown to contain during life a number of discrete spheroid bodies possessing an outer skin of a sudanophil, osmiophil, and methylene-blue-staining lipoid containing a relatively chromophobe but neutral-red-staining core. A yellowish, fat-solvent-soluble and relatively less osmiophilic material gradually accumulates within the centre of the spheroids to produce a mulberry-shaped mass, which may, however, retain some trace of the external skin attached to it. I identify the spheroid bodies as the true Golgi complement of *Helix* neurones, and the yellowish material as Golgi product.

The Mitochondria

Few workers would object to the classification of the structures stainable during the life of the cell by very dilute Janus green B as the mitochondria. These slender filaments or coccoid chains are the only morphological counterparts, visible in the living cell, of the batonnettes of earlier workers. The resemblance becomes strikingly apparent if the batonnettes are only partly bleached by the Verrati bleacher, whereby they are greatly reduced in thickness, and usually appear as a moniliform chain of coccoid particles (Text-fig. 7). Kolatchev himself (1916) observed that the mitochondria of *Helix* were linked to the batonnettes through a complete chain of intermediate forms, and he advanced the hypothesis that the batonnettes were derived from the mitochondria. Under the conditions of the Mann–Kopsch technique the slender filaments and coccoid chains are thickened by the deposition of successive layers of reduced osmium, which produces within the cell an apparently *de novo* structure, the batonnette. In most Mann–Kopsch preparations, besides the fully formed batonnettes, numbers of darkly impregnated smaller granules of various shapes and sizes occur. It is difficult to say exactly what these granules are, but possibly some of them are impregnated individual coccoid mitochondria.

The cytoplasm also contains numerous irregularly shaped flocculated masses, slightly impregnated by osmium and appearing light brown in colour. These masses can be seen after a wide variety of fixations and stains, and their occurrence in the Mann–Kopsch material and their frequent association with the batonnettes is not surprising. These flocculated masses when associated with the batonnettes are described as 'archoplasm' by some workers, but it is not at all uncommon to see many such masses, unaccompanied, in between the batonnettes and not attached to them.

Boyle (1937), on the basis of their reaction to the dye nile blue, concluded that the spheroids were 'metamorphosing mitochondria' in the process of swelling up to form lipoidal globules.

Mr. A. J. Cain (1947) of this Department, who has recently published a critical survey of the histochemical uses of nile blue, informs me that when this dye is used at the concentration employed by Boyle (1:10,000 in 0·75 per cent. NaCl) a blue reaction given by cellular granules cannot be interpreted as indicating the presence of lipoids. Under these conditions the dye will react simply as a basic dye and in my own experience it will give results very similar to methylene blue, staining the chromophil portion of the Golgi system only.

Blue staining with nile blue is completely unspecific (Lison, 1936): it can only be used as a basis for histochemical inferences if the body concerned is already known (through the use of sudan black or other colourants) to be lipoidal, in which case a blue colour does indicate the presence of lipines or fatty acids or both.

The Metaplasmic Granules

Legendre (1909) was one of the first investigators to describe the presence of granules of lipochrome within the nerve-cells of *Helix*. Gatenby (1923) and Gatenby and Hill (1934) confirmed this finding of Legendre but differed from Parat, claiming that they are stainable with neutral red but not with osmium tetroxide.

The term lipochrome is certainly a very ill-defined one from a histochemical standpoint. Gatenby thought that the granules might possibly be composed of lecithin.

I identify the lipochrome granules of Legendre and the lecithin granules of Gatenby with the mulberry forms of the spheroids (Text-figs. 1, 2, and 5). These forms are linked by a perfect series down to the smallest spheroid complexes, and studies with vital methylene blue indicate clearly that even the largest of these yellowish granules have attached to them a few granules of the chromophil portion of the Golgi apparatus. It seems clear that this yellowish material is formed within the chromophobe portion of the spheroids and finally, as the amount increases, it distorts the spheroid into an irregular mulberry mass. This transformation is probably associated with the rupturing or thinning of the outer chromophil envelope, which then becomes a discontinuous investment only retaining contact with the mass at a few parts of its circumference.

The methylene-blue-staining granules attached to the spheroids in *Helix aspersa* seem to correspond to those described by Young (1932) in cephalopod material. Young, however, observed aggregations of similar granules which he considered to be the Golgi apparatus completely free, grouped about the spheroids and unattached to them. I have not met with this appearance within living and vitally stained cells of *Helix aspersa*, but when ganglia are fixed and then stained either with iron haematoxylin, Azan, or Masson's technique groups of bright-staining granules, usually fuchsinophil, but sometimes staining with either the light green or the aniline blue, do occur grouped around characteristic clear spaces which are left in the cytoplasm as the ghosts of the spheroids when their lipoidal content has been dissolved in alcohols or xylene, previous to embedding. These bright-staining granules may be produced by fixation and staining of an external or 'extrasomal' Golgi product as has been described in *Mytilus* by Worley (1944).

Occasionally, too, masses of a clear pale-staining colloid are seen within fixed nerve-cells of *Helix*. Morphologically these fuchsinophil or iron-haematoxylin-staining granules and colloid masses are identical with those shown by Scharrer and Scharrer (1940) to occur within the nerve-cells of a number of invertebrates as well as vertebrates and interpreted by their school of workers as a secretory antecedent or precursor of a neurohormone.

The methylene-blue granules of the spheroid complexes appear to merge into the rims or crescents about the spheroids as staining progresses and it is possible that they represent a zone of maximal dye-concentrating power within the chromophil substances; for they are always the first to appear during progressive staining of the chromophil substance and the last to bleach when reduction of the dye to its leucobase commences.

The outer sheath or skin of the spheroid complexes when completely stained with methylene blue usually shows a striking similarity to the appearances produced by sudan black and osmium tetroxide vapour preparations, so much so that all doubt as to the rims, crescents, or caps exhibited by these methods being one and the same structure ceases to exist.

The central portions of the spheroids stain vitally with neutral red, irrespective of the presence of this endosomal product, and by definition comprise the vacuome of Parat. This author failed to recognize that this neutral-red-staining material is itself contained within a lipoidal capsule. From the researches of Duthie (1933), Hirsch (1939), and Worley (1946) it seems almost certain that the chromophobe spheroid is the main site for the collection of Golgi product (the endosomal product of Worley). In *Helix* the substance of the spheroids will occasionally stain a pink colour with nile blue at 1:10,000 in saline, and in addition it is possible to blacken it throughout its substance with osmium tetroxide according to the technique described on p. 455 of this paper. However, although the Golgi product itself seems on this evidence to be possibly lipoidal, it is certain that a limiting membrane consisting of lipines, and stainable with acid fuchsine after dichromate mordanting, can be very clearly shown to exist as a separate morphological component. This limiting membrane can only be identified with the chromophil zone of Worley, the Golgi externum of Hirsch, and the sudanophil dense lipoid-containing substance of Baker.

The Nissl Substance

No aggregations of material resembling the classical Nissl clumps of the fixed cell can be seen within living cells either by ordinary or phase-contrast microscopy.

In fixed mollusc tissue the Nissl substance has been studied by various workers. Garaieff (1909), Kunze (1921), Brambell and Gatenby (1923), Hanström (1928), and Boyle (1937) found the substance to consist of discrete granules distributed evenly throughout the cytoplasm. Erhard (1912) and Young (1932) with cephalopod material describe a homogeneous mass lying in the outer part of the cell.

My own findings indicate that this substance is most probably formed by coagulation of small discrete granules perhaps identical with the microneurosomes previously described in *Helix* (Thomas, 1947). Individual microneurosomes can be stained with both neutral red and Janus green, but they cannot be seen within the living cell unless the cell is slightly crushed by pressure of the coverglass. Apparently they are held within an optically similar gel during life, but with pressure the physical nature of the medium in which they are embedded changes to a fluid consistency in which the individual particles can move about freely. A concomitant slight alteration in the refractive index of the surrounding fluid enables the granules with their intense Brownian movement to be observed.

Actively moving microneurosomes can be coagulated on the slide by running fixing fluids under the coverglass, and the resulting globule of cytoplasm can be shown to stain strongly with the basic dyes usually employed to stain the Nissl substance.

The hypothesis that the microneurosomes are the physical basis for the Nissl substance would account for the observed fact that typical 'grumes' of the tigroid body cannot be seen within the living cell, and it would further account for the various forms this material may take in the same cell following different fixatives.

In conclusion I wish to thank Dr. J. R. Baker for his helpful advice and criticism throughout this study, and for taking the photomicrographs of Plate 1; also to Professor A. C. Hardy, F.R.S., for his kindness in providing me with such excellent accommodation within his department.

SUMMARY

1. Direct observation of the living nerve-cells of *Helix aspersa* reveals the presence of two categories of cellular organoids:

(*a*) Spheroid complexes consisting of a central core of chromophobe material covered wholly or in part by a chromophil lipine substance.

(*b*) Filaments and coccoid chains, and dispersed coccoid particles.

2. Evidence is put forward identifying the spheroid complexes as a dispersed type of Golgi apparatus and the filaments and coccoids as the mitochondria.

3. The chromophobe core of the spheroids is identified as the vacuome of Parat and is the site for the formation of an endosomal Golgi product (the lipochrome granules of earlier workers).

4. The so-called Golgi dictyosome and archoplasm cannot be observed in living cells and are probably an artifact of technique produced by an over-impregnation of the mitochondria with silver or osmium.

The drawings have been made with the assistance of a camera lucida. The photomicrographs were taken by Dr. J. R. Baker with a technique described by him in a special article on photomicrography in the *Journal of the Royal Microscopical Society*, vol. 62, p. 112. He used a Reichert 2 mm. fluorite immersion lens and Watson 14 Holos eyepiece.

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Further Remarks on the Histochemical Recognition of Lipine

BY

JOHN R. BAKER

(From the Department of Zoology and Comparative Anatomy, Oxford)

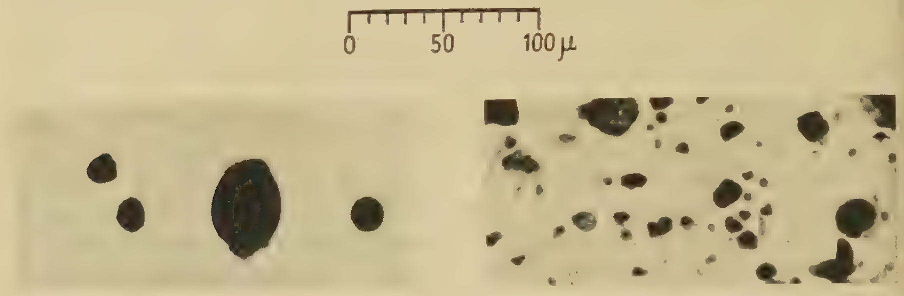
With one Text-figure

IN an earlier paper (Baker, 1946), a technique was described for the histochemical recognition of lipine. This technique was called the acid-haematein test. It was shown that lipines, after appropriate fixation and subsequent mordanting with potassium dichromate, display a special affinity for haematein, with which they react to give a blue, blue-black, or grey colour. If certain precautions are taken, lipines can thus be distinguished from other substances occurring in the tissues of plants and animals. It was found, however, that the crude galactolipine used in the investigation reacted much less strongly than lecithin, cephalin, and sphingomyelin. It will be recollected that galactolipine differs from these other lipines in containing no phosphoric acid radicle. The suggestion was made that if the galactolipine used in the test had been completely free from contamination with sphingomyelin, it might have proved negative to the test. If so, the acid-haematein test would be a test not for lipines in general, but for lipines other than galactolipines; that is to say, it would be a test for phospholipines. It is the purpose of the present paper to report the reaction of purified galactolipine.

In the earlier work, the lipines were all prepared by Weil's (1930) method. In the present investigation, crude galactolipine was prepared from the brains of sheep in the same way, but it was then purified according to the method of Rosenheim (1913). It was deposited on cigarette-paper, as before, and subjected to the acid-haematein test. The result was negative, for the colour given was not blue, blue-black, or grey, but yellow, tinged with greenish-brown. Other lipines, subjected simultaneously to the test as controls, gave positive reactions. The colour given by the galactolipine developed to a considerable extent in the hot potassium dichromate solution, before treatment with the dye began.

It was thought desirable to study the reactions of small globules of the various lipines to the acid-haematein test. To achieve this, each lipine was mixed with a thick sol of celloidin in 1:1 alcohol-ether, in the proportion of about 1 gm. of lipine to 10 c.cm. of celloidin sol. The lipine was suspended (or partly dissolved, in the case of lecithin) by stirring with a glass rod, and the cellodin was then caused to set into a gel by exposure to the vapour of chloroform. A small piece of the gel was cut out and subjected to the acid-haematein test. The same technique was used as though the celloidin had been a piece of tissue, except that a sliding microtome was used and thicker sections were cut. Microscopical examination revealed that in each case the lipine was in the form of small globules.

The results of these tests are illustrated by Text-fig. 1. The exposure and development of the negative and positive were carried out in exactly the same way in each case, so as to make the photographs comparable. The egg-lecithin globules were blue throughout, though sometimes unevenly dark at different distances from their surfaces. Cephalin (from the brains of sheep) gave a different appearance. The centre of each globule was yellow, while the whole of the outer part, or often a layer a little below the surface, was stained blue-black (though some of the globules were yellow all through). A number of the globules were cracked across; when this was so, the cracked surface had an affinity for the dye. Sphingomyelin (crude, from the brains of sheep) gave a different appearance again. The small globules were stained blue or blue-black, while larger ones were yellow in their centres and only had a blue-black rim. The globules of purified galactolipine, which were more uniform in size than those of the other lipines, gave scarcely any trace of a positive reaction. They appeared yellow, sometimes with an extremely faint tinge of blue. When viewed in optical section, their periphery appeared as a very thin dark line, but this coloration could not be recognized when the upper or lower surface of a globule was focused.



Lecithin Sphingomyelin

Cephalin Galactolipine

Text-fig. 1

To find the cause of the yellow colour seen in the globules, all four lipines were subjected to a test differing from the standard one in the omission of any treatment with haematein: the sections were treated with 2 per cent. acetic acid instead of the dye solution. In each case the globules now appeared yellow. The darkening of the globules to a yellow colour seems to have occurred mainly during the treatment of the celloidin blocks with hot potassium dichromate.

The results recorded in this paper show that the acid-haematein test, controlled by the pyridine-extraction test, is a method for the histochemical recognition of phospholipine.

Note. The borax-ferricyanide solution used in the acid-haematein test need not be kept in the refrigerator, as was stated in the earlier paper. It suffices to keep the bottle in the dark when not in use.

I thank Prof. A. C. Hardy, F.R.S., for the provision of all the facilities required for the work described in this paper, and Mr. A. J. Cain for the benefit of many helpful discussions.

SUMMARY

1. Carefully purified galactolipine has scarcely any tendency to react positively to the acid-haematein test.

2. It follows that the acid-haematein test (controlled by the pyridine-extraction test) is a method for the histochemical recognition not of lipine in general, but of phospholipine.

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An Examination of Baker's Acid Haematein Test for Phospholipines

BY

A. J. CAIN

(*From the Department of Zoology and Comparative Anatomy, Oxford*)

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INTRODUCTION

THE only histochemical test for lipines that was regarded as specific by Lison (1935, 1936) is that of Smith-Dietrich. Baker (1946) has criticized it on the grounds that it is inadequately specified, not very sensitive, and gives a negative reaction with pure lecithin. He proposes his acid haematein test, based on the Smith-Dietrich test but with a pyridine extraction control, as being free from these defects. He has since shown (1947) that the test is for phospholipines only.

It is important to try to assess the value of this test. So many 'histochemical' tests have been shown to be unspecific, and the results obtained by them thereby invalidated, that caution is necessary in admitting the specificity of a new one. It would be better to have no tests rather than unreliable ones.

This paper presents the results of an examination of Baker's test, and gives reasons for believing that, provided only a definitely positive result is considered, it is specific, though there are certain possible sources of invalidity, as Baker has pointed out.

MATERIALS AND METHODS

Transverse sections of the rhynchobdellid leech *Glossiphonia complanata* (L.) were found to give a good variety of tissues, with plenty of lipine in the fat-cells. Liver, kidney, testis (for interstitial cells) and small intestine of the mouse, and adrenals of the rat were also used. In addition, tests were made on paper impregnated with various pure substances and mixtures. Some of these were repeats of Baker's (1946) tests; all were carried out in accordance with the instructions given by him, except that in some cases the paper was soaked in the melted substance or mixture, and was therefore very thickly coated.

The method employed was to determine what results were given by the acid haematein and pyridine extraction tests when performed in exact accordance with Baker's instructions, and then study the effect of varying the chemical and physical components of the tests. For controls, sections and papers were coloured with sudan black.

Diphenyl-carbazide was used for detecting chromium.

Results and Discussion

Standard Results obtained with the Acid Haematein and Pyridine Extraction Tests

For convenience, these tests will be referred to as the AH and PE tests respectively. A blue, blue-black, or black coloration given by AH but not by PE indicates phospholipine.

The first point that is noticed on examining an AH section of *Glossiphonia* is that the nuclei, except for the plasmosomes, are nearly colourless. The mitochondria of the nephridial cells, the Golgi apparatus of the stomach epithelial cells, and usually the whole of the cytoplasm of the intestinal cells are blue, as are the cytoplasm of the fat-cells, and granules in the muscle-cells and in certain corpuscles in the coelomic spaces. The epidermis (except for the mucous cells) and the dermis are blue-black or black. Some of the dorsoventral and oblique muscle-fibre cortices may be blue also, but those of the longitudinal fibres vary from colourless through yellow to brown. The interfibrillar spaces in the longitudinal muscles are not usually coloured. The nerve-cords are pale blue, with dark blue in the nerve-cells. The cytoplasm of the salivary gland cells is very dark blue; the pigment cells remain pale brown, as in unstained sections. The cuticle is pale yellow.

Contrasts are seen immediately on examining a PE section. The nuclei are now full of small granules coloured a dark blue-grey. All the blue colorations mentioned above have disappeared completely or have been replaced by a faint blue-grey, except that the epidermis and dermis remain blue-black or black as before. Many of the interfibrillar spaces, particularly near the dermis, are now black or very dark brown. The cortices of the dorsoventral fibres are yellow or dark brown, those of the longitudinal ones, seen in cross-section, may be yellow, brown, black, brown with blue shadings, or deep blue. In one section they may all be yellow; in another, in each bundle there may be some yellow and some dark blue or dark brown. It is possible that the colour may vary with the degree of contraction. The pigment cells are now full of dark blue granules smaller than those seen in AH and unstained formal-calcium preparations. The nerve-cords still contain some blue. The general tone of the cytoplasm is pale yellow, usually slightly darker than in AH preparations; the cuticle remains pale yellow.

It is evident, then, that in transverse sections of *Glossiphonia* we have examples of all the four classes of substances distinguished by Baker, thus:

(A) AH positive, PE positive. Epidermal cells, plasmosomes.

(B) AH negative, PE positive. Chromatin, pigment granules, interfibrillar matrix.

(C) AH positive, PE negative. PHOSPHOLIPINES. Cytoplasm of fat-cells, salivary gland cells, and intestinal cells, mitochondria of nephridial cells, Golgi apparatus of stomach epithelial cells.

(D) AH negative, PE negative. Fat droplets, cuticle, cytoplasm of nephridial and other cells; and cortex of muscle cells frequently.

The Stages of the Tests

The two tests may be summarized as follows:

AH. (i) Fix in formal-calcium, a non-precipitant fixative specially designed to prevent the loss of lipines (Baker, 1944).

(ii) Soak in potassium dichromate-calcium chloride solution at room temperature and then at 60° C., to make the phospholipines insoluble and mordant them. Wash well in water.

(iii) Cut frozen sections at 10 μ. (Embed in gelatine if necessary.)

(iv) Mordant again in the same fluid at 60° C. for an hour only, as the chromium compound left in the tissues to act as mordant may be washed out of small particles of lipine (Baker, 1946).

(v) Stain in acid haematein for 5 hours at 37° C.

(vi) Differentiate in borax-ferricyanide for 18 hours at 37° C.

(vii) Mount in Farrants's medium.

PE. (i) Fix in weak Bouin to render the phospholipines easily extractable by solvents and increase the power of proteins to give a positive reaction (Baker, 1946).

(ii) Wash in alcohol to remove picric acid.

(iii) Soak in pyridine, first at room temperature then at 60° C., to remove phospholipines (and all other lipoids).

(iv) Wash in water, then go to stage (ii) of the AH test and proceed as for it.

On consideration of these summaries the following questions arise:

(*a*) A calcium-haematein lake is easily produced by adding calcium chloride solution to some acid haematein, alkalinizing, and filtering off and washing the precipitate. A purplish-blue solid is obtained, which turns rusty red on prolonged exposure to the air. It is insoluble in borax-ferricyanide but dissolves readily in acid haematein. Do the calcium ions in the fixing and chroming solutions assist in mordanting?

(*b*) What evidence is there that a chromium compound is left in the tissues?

(*c*) What is the degree of completeness of extraction by the PE method?

(*d*) Why do some substances stain after PE but not after AH? Obviously something more than a mere extraction has been performed.

(*e*) Does the specificity of the test depend on the period of chroming or on a reaction peculiar to phospholipines?

To which we may add after considering the results described above:

(*f*) How are both blue and brown colorations produced in the finished preparations?

Investigation of the Tests

(*a*) *Role of the Calcium Ions in Fixation and Mordanting.* Variations of the AH test as shown below were tried out to determine the effect of the omission of dichromate and calcium at different stages. All were done on *Glossiphonia* sections.

Variation	*Result*
1. Fix in 4 per cent. formaldehyde, and omit chroming and differentiation.	Whole section dark reddish-brown. No blue.
2. Same as (1) but with differentiation.	General pale yellow-brown. No blue.
3. Fix in formal-calcium, omit dichromate from the chroming solution, omit differentiation.	General brown. Phospholipine in fat-cells slightly darker brown. No definite blue.
4. As (3) but with differentiation.	Pale brownish-yellow throughout. No blue.
5. Standard AH but with calcium chloride omitted from the chroming solution.	As standard AH results above, but blue reduced greatly. Positive results only in bases of intestinal cells and cytoplasm of a few fat-cells.

The last variation was tried on other tissues also. With rat adrenal, considerably less blue was seen than in standard preparations. With mouse small intestine there appeared to be no reduction in blue-staining, and with mouse liver it was slight.

The conclusions from these results are that:

(i) dichromate is necessary for blue and almost all brown staining in the finished preparation,

(ii) it is necessary for the production of fast browns, and

(iii) calcium ions are necessary with dichromate for the maximum blue staining in some tissues.

Also, the behaviour of various lipoids in thick deposits on paper was investigated in the same way. The following results are representative:

Mixture	*Procedure*	*Colour at start*	*Colour after staining but before differentiation*	*Colour after differentiation*
Pinene saturated with cholesterol at 60° C.	Standard AH.	White.		General brown. Edges of some crystals medium to dark blue. Scattered dark blue granules.
	AH but omit calcium chloride from chroming solution.	White.	Very pale brown. A few small blue granules.	Pale greenish-brown, no blue.
	AH but omit dichromate.	White.	White.	White.
	AH but omit calcium chloride throughout.	White.	Very pale brown. A few small blue granules.	General pale greenish-brown. A few dark brown granules. A faint lilac tinge on some crystals.
	AH but omit dichromate, and calcium chloride from fixative.	White.	White.	White.

Exactly comparable results were obtained with an equimolecular mixture of cholesterol and oleic acid (BDH redistilled) and with oleic acid freed from all traces of phosphorus. In all cases, after the omission of dichromate no coloration (or only the faintest trace) was seen at any stage, but omission of calcium chloride had no effect. With these mixtures there is no loss of lipoid if calcium is omitted, as there is with phospholipine; so we may conclude that the calcium ions play no part in mordanting here, and it is very improbable that they do with phospholipines.

Examination of formaldehyde-fixed *Glossiphonia* sections with sudan black shows that a considerable amount of lipoid, including some phospholipine, is preserved without treatment with dichromate. Fixation is, of course, rather poor. It is not possible to say from examination of sections that less phospholipine is preserved after 4 per cent. formaldehyde than after formal-calcium, but it is seen from variation (5) on *Glossiphonia* and rat adrenals above that omission of calcium chloride from the chroming fluid does allow phospholipine to escape from some tissue, though not completely. It appears that the short fixation (6 hours) in formal-calcium is enough to fix proteins but not enough to affect the solubility of phospholipines if they are exposed to fluids at 60° C. Baker (1944) found in his studies of lipine solubilities that formaldehyde does make lipines less soluble in various solvents after 24 hours' fixation. One suspects that some lipine is lost even at room temperature if plain formaldehyde is used. If two pieces of lecithin paper prepared by Baker's method (1946) are fixed for 6 and 29 hours respectively in 4 per cent.

formaldehyde, left overnight in water at room temperature, and coloured with sudan black, it is seen that much more lecithin remains on the paper after the longer fixation. Little or none remains on paper fixed for 6 hours and soaked in plain dichromate solution at 60° for 24 or for 8 hours.

We may conclude that:

(i) under the conditions of the AH test neither formaldehyde nor calcium chloride, separately or in combination, will fix phospholipines, and

(ii) the calcium ions merely restrain the phospholipines from passing into solution. (It was for this purpose they were added to the fixing and chroming solutions by Baker.)

(*b*) *Evidence for a Chromium Compound in the Tissues.* It is seen from variations (i) and (iii) in section (*a*) above that a dark brown coloration can be produced in the tissues by acid haematein without the aid of dichromate, but it is reduced to a pale yellow-brown by differentiation; no blue is seen. Potassium dichromate is essential for the formation of the fast blue and brown colours; but a chromium lake is not producible by the method described above (part ii) for the calcium lake. Does the dichromate act as a mordant, or are the fast colours produced by the dye and oxidation products in the tissues?

Sections of *Glossiphonia* were prepared (*a*) as for AH and (*b*) with formal-calcium fixation but no chroming. Diphenyl-carbazide in 90 per cent. alcohol was used for the detection of chromium as anion. This substance gives colours varying from red to violet with several metallic cations and chromates, arsenates, molybdates, phosphates, and similar anions. (For an account and references, see *BDH Reagents for Delicate Analysis including Spot Tests*, 7th ed. 1939, BDH, London.) The unchromed sections were used as controls. Each section was washed in distilled water, laid on a slide, covered with one drop of a saturated solution of diphenyl-carbazide in 90 per cent. alcohol, and examined at once. (The reagent, if left exposed to the air, gradually goes pink by itself.)

Neither the controls nor the chromed sections gave any colour with the reagent applied alone. On acidifying with acetic acid, the controls still gave no colour, but a fine red was seen at once in the chromed sections. The coloured substance, unfortunately, is alcohol-soluble, and diffuses out rapidly, but on examining the section immediately after applying the reagent it was seen that the colour was most intense just where the deepest staining was seen in AH sections, that is, in the epidermis, intestine, fat-cells, and nerve-cord.

It appears then that a chromium compound, unstable in very acid solution, is to be found in the tissues, most plentifully where the staining is densest. The acid of the dye may have a slight differentiating effect on the mordant, perhaps.

As a confirmation, a paper test on lecithin and caseinogen, both of which give deep blue with AH, was carried out, unimpregnated paper being used as the control. All the papers were put through the AH test without sectioning, the acid haematein being replaced by 2 per cent. acetic acid. After 24 hours' washing, both the lecithin and caseinogen papers gave strongly positive results and the controls were colourless. Washing for 2 more days reduced the colour in both cases but did not abolish it. (These tests might perhaps be criticized on the grounds that as it took 24 hours' washing before the controls were free from chromium compounds some chromium may have been trapped inside the papers under the layers of lecithin or caseinogen; the reduced reaction on further washing is not necessarily evidence that the chrome-lecithin or chrome-caseinogen compound is removed by washing.)

Pieces of bath-sponge stain deep brown after both AH and PE. After AH with no mordanting they stain deep brown but lose almost all their colour during differentiation. Mordanted pieces give a strongly positive result with diphenyl-carbazide and glacial acetic acid, but slowly. Also, if lecithin paper, unchromed, is stained in acid haematein, most of the lecithin is lost and there appears to be no staining. If acid haematein made up with 1 per cent. calcium chloride instead of distilled water is used, there is still no staining and little if any lecithin is lost.

Apparently a chromium compound is part of both blue- and brown-coloured substances. (The blue colour with dichromate and lipoids has, of course, been considered a chrome lake for very many years.) The above experiments do not rule out the possibility of the chromium combining with oxidation products of the lipoids rather than with the lipoids directly. Smith and Mair (1909) have shown that oleic acid will form an intermediate chrome compound in the course of oxidation to a dioxystearic acid.

(*c*) *Extraction by Pyridine*. The test of this is very simple. A *Glossiphonia* section was produced as for the PE test but was coloured with sudan black instead of acid haematein, and compared with an AH section similarly treated. The result shows that the extraction is very good. An exceedingly faint coloration is visible in places where phospholipine was very plentiful (notably in the fat-cells), and the rest is uncoloured. The contrast with the sudan-coloured AH section is most striking.

(*d*) *Substances staining after PE but not after AH*. From what has been said so far it is understandable that substances should be AH +ve and PE −ve, Ah +ve and PE +ve, or AH −ve and PE −ve, but it is a little difficult to see how the combination AH −ve and PE +ve can occur. The differences between the two tests are:

(i) the use of acetic and picric acids in the PE fixative;

(ii) the omission of calcium chloride from the PE fixative; and

(iii) the use of the strong base pyridine in the PE test for extracting lipoids.

This subject was not followed up in detail, but some points should be mentioned.

In *Glossiphonia* sections prepared as for PE but with the actual extraction omitted, the pigment-cell granules are much smaller and darker than in AH sections, and nuclei, having been treated with a powerful protein precipitant, show dark granules of chromatin. It seems likely that in these cases the difference between AH and PE results is due to non-precipitant and precipitant fixation respectively. Baker has noted (1944) that chromatin in thick layers is coloured by AH. The colour of the pigment cells is probably entirely intrinsic and not due to staining.

The case of the interfibrillar matrix is more puzzling, but it should be noted that in exceptional instances it is coloured after AH. It is invariably coloured after PE but to a depth varying greatly in different individuals. In PE-fixed sections without extraction and chroming the pigment-cell granules are very dark (which confirms that their coloration is by concentration of their own pigment) but the rest of the section is a general yellow-brown, as in a PE section with only the extraction omitted. This seems to mean that the pyridine has some effect on the interfibrillar matrix, but the results vary in different individuals.

(*e*) *Specificity and the Period of Chroming.* Smith and Mair (1909) have investigated the action of dichromate solutions on lipoids, and their results are of great interest here. They found that many unsaturated lipoids would give a blue colour with haematoxylin after chroming for various periods, and even saturated ones could do so if mixed with cholesterol, which by itself was negative. The necessary period of chroming for unsaturated lipoids was shortened by adding cholesterol to them, in a few cases to 24 hours. With lecithin they obtained a negative result, almost certainly because, as Baker has suggested, there being no calcium ions present it went into solution. Kaufmann and Lehmann (1926), on examining the Smith-Dietrich test, considered it specific if a black colour only were taken as positive; blues, greys, and browns were given by various mixtures not containing lipines. Baker reduced the concentration of the stain considerably so that these weaker, non-specific results might be abolished. Two of Kaufmann and Lehmann's mixtures containing cholesterol or cholesteryl oleate but no lipines gave positive results, and Lison (1936) concludes that unless cholesterol or cholesterides can be excluded there remains a slight doubt. Smith and Mair found that a cholesterol-oleic acid mixture gave a clear blue after 24 hours' chroming at 65° C. with saturated potassium dichromate solution.

The following lipoids were therefore investigated, thick coatings on paper being used:

Oleic acid (phosphorus-free).
Cholesterol and oleic acid (BDH redistilled) in equimolecular proportions.
Cholesterol dissolved to saturation in pinene at 60° C.
Cholesterol dissolved to saturation in tributyrin at 60° C.

Details of the cholesterol-pinene results are given in part iii above. Cholesterol by itself is uncoloured by AH, but is solid at the temperatures of the test. All the above gave a few granules of pale or medium, and sometimes dark blue. Occasionally edges of thick masses of crystals were tinged bluish or lilac. In the case of the oleic acid, staining produced a definite purple or purplish-blue which was almost completely removed during differentiation.

As was mentioned above, Kaufmann and Lehmann found that very many mixtures gave faint colours after the Smith-Dietrich test. Unfortunately, consideration of their results with nile blue (Cain, 1947) shows that some of their substances, including triolein or cholesterol in one case, were not pure. (It is worth noting here that their specimens of phrenosin and kerasin gave heavily positive results in mixtures containing no other lipine, and faint ones by themselves, with the Smith-Dietrich test. Baker has shown (1947) that a mixture of phrenosin and kerasin carefully freed from all traces of sphingomyelin (the most difficult of the phospholipines to remove) is negative to AH.) Consequently, their detailed results must be accepted with reserve, though supported in general by Smith and Mair's results and by those given above. At the same time, it must be remembered that they were the first to use extensive tests on pure substances and mixtures to assess the validity of histochemical methods for lipoids.

The weak colorations obtained with AH in mixtures on paper were never seen throughout the coating, and this was very thick, probably of the order of 100 or 200 μ, whereas the coloration of lecithin paper prepared according to Baker's instructions is present wherever there is lecithin on the paper and is quite a dark blue, although the total thickness is only about 10 μ (Baker, 1946). Very occasionally, as in a cholesterol-tributyrin preparation, there is a small cloud of granules of a medium blue but again only locally in a mass of the mixture far larger than could be expected in tissues. After study of the results it is concluded that a definitely positive result (dark blue or blue-black) with AH but not with PE does indicate phospholipines, but that with weak results (pale blues and greys) there is no certainty, unless, of course, a chemical analysis of extracts of the tissues shows that phospholipines are the only lipoids present. The intense coloration of mitochondria in many cells gives a good example of a positive result in very small bodies.

Prolonged chroming has been shown by Smith and Mair to produce blue colours with haematoxylin in many lipoids. (However, on repetition of one of their results, it was noted that chroming of the cholesterol-oleic acid mixture for several days gave with AH an increase in brown coloration but not in blue.) The specificity is therefore dependent on the period of chroming, which must never be increased. With AH, decreasing the period caused an increased response in the mitochrondria of mouse liver but a decreased one in those of mouse small intestine and kidney, and appeared to have no effect on the pale blues of the cholesterol-oleic acid mixture. It is therefore not recommended, as it may weaken the results obtained with some tissues and make them uncertain. A preliminary extraction with acetone to remove interfering lipoids was tried and gave fairly good but definitely fainter results and considerably increased the length of the test.

(*f*) *The Blue and Brown Colorations.* It was shown above (*b*) that a chromium compound is formed in the tissues in both blue- and brown-staining. If an undifferentiated *Glossiphonia* section is examined, it is seen that what is blue in the differentiated section is blue there also and in much the same depth of colour. Differentiation, even for 88 hours, makes little difference to a *Glossiphonia* section as far as the blue stain is concerned. This is not true of the brown. In the undifferentiated section all that is not blue is dark brown except ground cytoplasm, which is nearly colourless or pale yellow. The effect of differentiation is to reduce the dark brown to pale yellow-brown or yellow. After 88 hours' differentiation, plasmosomes in the nuclei of intestinal cells have changed from black to brown. If, however, the progress of the AH test is observed with pure oleic acid (see (*e*) above) it is seen that a blue or purple colour is produced which is removed by differentiation. Consequently, it is important to keep to the prescribed period of differentiation. Shortening is not permissible; lengthening seems to have little effect on phospholipines in general, but might remove the colour from very small objects or weaken it to the point of uncertainty.

It will be noticed from Baker's results of paper tests that all the very dark blue-staining substances except legumin contain phosphorus. It is quite probable that such substances as fibrinogen, blood-albumen, mucin, nucleoprotein, nucleic acid, and perhaps trypsin might be impure. A faint positive result might almost be expected with blood-serum (rabbit) and blood-plasma (fowl). However, sodium glycerophosphate gave a negative result. But this substance dissolves in both formal-calcium and dichromate-calcium, so it is likely that it was lost from the paper at an early stage and the negative result is not significant. A negative result with a paper test means nothing unless it is shown that the substance was still present at the end of the test or that it would have been lost from tissues. A small quantity of this salt was dissolved in dichromate-calcium and the solution incubated at 60° C. for 18 hours as for chroming for AH. At the end of that time a crystalline precipitate had formed which was removed by filtration, when it was seen to be a very pale yellow, almost white. It was only slightly soluble in water, and a small portion tested with diphenyl-carbazide gave a faint red at once which was greatly increased on acidifying. (The first faint colour was almost certainly due to the retention of a little mother-liquor.) The rest was stained and differentiated as for the AH test. The resultant colour was black. This substance resembles the blue-stained substances in AH sections quite closely in its formation. One might suggest that rapid and intense blue staining is usually due to some combination between chromium and substituted phosphoric acids.

But this will not explain the case of legumin, and would lead one to suppose that at least some nucleoproteins would stain heavily, which is not so. Baker notes that all three dark-blue staining proteins (caseinogen, mucin, and legumin) are very acidic. Stearic, palmitic, and ricinoleic acids all gave a negative or exceedingly faint positive result, so the mere presence of an acidic group is not sufficient. Smith and Mair's work with oleic acid appears to show that chromium enters into combination at the double bond; prolonged chroming abolishes the capacity to stain, a dioxy-compound being formed which contains no chromium.

The only safe conclusion appears to be that with the AH test, blue and brown colours are produced, both fast to 18 hours' differentiation with borax-ferricyanide at 37° C. The dichromate acts as a mordant in the blue staining and some at least of the brown, though it is not proved that in some cases it may not cause oxidation first. Under the conditions of the AH test mordanting takes place most readily with certain acidic compounds, especially some containing phosphorus. (This suggests that interference might come from bound phosphorylated sugars and other compounds in tissues if they are removed only by the action of pyridine.)

The production of different colours according to the substrate by the same dye *and mordant* under the same conditions has not, to my knowledge, been emphasized before, and seems worth investigation.

My thanks are due to Dr. J. R. Baker, who has supervised this work and discussed many of his unpublished results with me.

SUMMARY

1. Baker's acid haematein test for phospholipines is specific provided that only a definite positively result is considered. Very pale blues and greys may be caused by other lipoids, which if present in very large masses may possibly show medium to dark blue granules but will not be coloured all through.

2. The mechanism of the test appears to be as follows:

(*a*) Phospholipine is not fixed by formal-calcium but is restrained from passing into solution by the calcium ions, which play no other part.

(*b*) Phospholipine combines readily with chromium from the mordanting fluid, and is then rendered insoluble and mordanted. Other substances, acidic and usually containing phosphorus, are mordanted as well.

(*c*) On staining, blue and brown colorations are formed; in both cases the dye attaches itself to the chromium in the various substrates.

(*d*) On differentiation, some browns and most blues, particularly those with phosphoric substrates, remain nearly fast, but most browns and the weak blues of certain lipoids (not phospholipines) are greatly reduced or removed entirely. The period of differentiation must not be shortened.

(*e*) Blue-staining lipoids (phospholipines) are distinguished from other blue-staining substances by an extraction with the lipoid solvent pyridine, after special fixation. The other substances, and any bound lipoid not removable with pyridine, remain.

3. Since the specificity of the test depends on the *relatively* greater affinity of phospholipines among lipoids for the mordant, the period of chroming must not be lengthened.

4. One reason why some substances are coloured after pyridine extraction but not after acid haematein is that in the former case they are precipitated and so concentrated; in the latter they are not. This is not a general explanation for the whole class of such substances.

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A Comparative Histological Study of Haemosiderin in the Uteri of Mice of Cancerous and Non-cancerous Strains

BY

VERA E. JONES, Ph.D.

(*From the Department of Zoology, University of Liverpool*)

With 3 Text-figures and 3 Plates

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INTRODUCTION

WITHIN the uterine wall in mice large masses of a brown or yellowish-brown granular substance are often observed. This has been identified as haemosiderin—a pigment derived from the breakdown of haemoglobin. This substance contains iron and therefore gives the well-known 'Prussian blue' reaction on treating with potassium ferrocyanide and hydrochloric acid.

In the course of the comparative histological examination of uteri of mice of cancerous and non-cancerous strains, it soon became apparent that, although masses of haemosiderin occurred in the uterus of almost every mouse of the cancerous strain, such large masses were not observed in the uteri from the non-cancerous strain. Further study, however, showed that the pigment is formed in both cancerous and non-cancerous strains from the blood which is extravasated into the tissues of the uterus at parturition, but in the cancerous strain it is formed somewhat more slowly and persists much longer than in the non-cancerous strains examined. The evidence for these differences, their nature, and their possible significance form the subject-matter of the present paper.

MATERIAL AND TECHNIQUE

The strains of mice employed in this investigation included cancerous and non-cancerous strains. The cancerous strain—the R III albino strain of Dobrovolskaia-Zavadskaia—showed a very high incidence of mammary carcinoma, in females over 7 months old, at the time of investigation (1940). This strain was bred in this department as two sub-strains, both cancerous, the CB and CBB strains. Another albino strain, non-cancerous, the Wistar strain, has been inbred for over 4 years in this department without ever developing cancer. A recent non-cancerous strain, of piebald mice, the P strain, has been inbred for almost 5 years, again without showing any cancer development. The PC strain, also piebald, and the XL strain, self-fawn and self-brown, have both been entirely cancer free during the 3 years that they have been inbred in this department. All the uteri used for study have been fixed in either Bouin's fluid or formol-saline solution (9 per cent.). In order to prevent muscular contraction of the uterine walls the whole female reproductive apparatus was dissected out and pinned, in a stretched position, on a layer of paraffin wax in a shallow dish into which the fixing fluid was then poured. After fixation, dehydration, and clearing, the right and left horns of the uteri were embedded separately.

Microscopic sections were cut 5μ in thickness, and stained, for ordinary histological study, with Ehrlich's haematoxylin and eosin. Alternate slides of the serial sections were treated with potassium ferrocyanide and hydrochloric acid, after prolonged contact with nitric acid alcohol, and then counterstained with eosin. The details of this method are given by Bolles Lee (1937) and are as follows: The sections, after removal of the wax, are placed in nitric acid alcohol (3 per cent. in 95 per cent. alcohol) for 36 hours at 35° C. They are then washed in 90 per cent. alcohol and in distilled water. After washing, they are placed, for not more than 5 minutes, in a freshly made solution composed of equal parts of 1·5 per cent. potassium ferrocyanide in water and 0·5 per cent. hydrochloric acid, also in water. The sections are again washed carefully in distilled water, stained for 3 minutes in eosin (1 per cent. in 30 per cent. alcohol), differentiated in 90 per cent. alcohol, cleared in cedarwood oil, and mounted in benzene balsam. By this method haemosiderin is stained a bright Prussian blue.

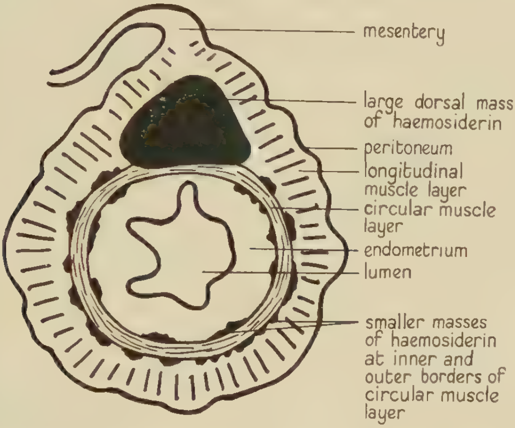
DATA

Description of Haemosiderin and its Location in the Uterus

In transverse sections of some uteri a large mass of brown or yellowish-brown material is seen situated dorsally in the thickness of the wall just below the mesentery (Text-fig. 1). Serial sections reveal that these dorsal masses are present only at intervals.

Occasionally there are also in the uterine wall smaller scattered clumps of the material situated at the inner and outer borders of the circular muscle layer. Such scattered material has been observed in only a few of the uteri examined and has not been included in the present study. Where it occurs at all it is present all along the uterus, not at intervals as are the dorsal masses. It has never been seen in uteri which do not contain dorsal masses, and in only a very small proportion of those that do.

Under the high power of the microscope the material is seen to be granular in consistency, the granules being arranged in small ovoid groups or clumps (Pl. I, figs. 1 and 2) which tend to aggregate to form larger masses (Pl. I, fig. 2). These small ovoid groups appear to be cell-bodies crowded with the granules, since each contains a nucleus (Pl. I, fig. 1). The granular material in form and natural colour agrees with the published descriptions of haemosiderin, and on staining with potassium ferrocyanide and hydrochloric acid it gives the typical Prussian blue reaction.


mesentery
large dorsal mass of haemosiderin
peritoneum
longitudinal muscle layer
circular muscle layer
endometrium
lumen
smaller masses of haemosiderin at inner and outer borders of circular muscle layer

TEXT-FIG. 1. Diagram of a transverse section of mouse uterus a few months after parturition, to show distribution of haemosiderin.

Origin of the Pigment

As haemosiderin is known to be the pigment commonly formed when there has been extravasation of blood into the tissues, it was thought that possibly the haemosiderin present in the uterine wall is derived from the blood extravasated at parturition. The large masses of haemosiderin are found only in the dorsal wall of the uterus, close to the mesentery—a location identical with that of the placentae (cf. Pl. II, figs. 3 and 4); the masses occur at intervals along the uteri, as do the placentae; these masses were never found in virgin uteri, and when the breeding records were examined for the animals under investigation it was found that in every case where these masses of haemosiderin occurred the animal had borne young. Finally, by examining a

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PLATE I

Fig. 1. T.S. of uterus of mouse (of cancerous strain) showing haemosiderin within phagocytes. Ehrlich's haematoxylin and eosin. Oil immersion. × 900.

Fig. 2. T.S. of uterus of mouse (of cancerous strain) showing massing together of pigment-carrying phagocytes. Fe and eosin. High power. × 600.

complete series of sections the number of masses of haemosiderin could be counted, and on referring to the breeding records it was found that, where only one litter had been produced, the number of masses of haemosiderin corresponded in every case with the number of young in the litter. It was then found that these dorsal masses of pigment could be seen with the naked eye as dark spots situated close to the mesentery when the whole uterus was cleared in cedarwood oil (Pl. II, fig. 5). (Uteri to be examined in this way should be fixed in formol-saline or formaldehyde as these clear much more perfectly than Bouin material.) The number of these dark spots in every case corresponded exactly to the number of young born, when the mouse had had only one litter. Uteri taken immediately or very shortly after parturition also show similar dark spots when cleared. Pl. II, fig. 7, shows a uterus, from the non-cancerous strain, taken 2 days after parturition, and Pl. II, fig. 8, a cancerous strain uterus also 2 days after parturition. When sectioned, however, these spots are found to contain very little haemosiderin, if any. Each is composed of a mass of extravasated blood and torn tissue which marks the former site of the placenta. These spots are very large, but by 25 days they are much smaller, as can be seen in Pl. II, fig. 5, which shows a cancerous strain uterus taken 25 days after parturition. This decrease in size is caused by a healing of the torn tissues. Sections then show that it is chiefly a quantity of haemosiderin which marks the site of the placentae. Later, uteri from mice which had been pregnant more than once were examined, and it was often found that the sites of the placentae of successive pregnancies could be distinguished from one another by the different size of the dark spots, since the placentae of one pregnancy did not overlap with those of another. The dorsal masses, when present, from two successive litters could always be distinguished when only a few days had elapsed since the last pregnancy, as seen in Pl. II, fig. 6. This is a photograph of the cleared uterus of a mouse of the cancerous strain, 3 days after parturition, which has had two litters with seven young in each. In the photograph, seven small dark spots and seven larger ones are to be seen situated close to the mesentery. Here the seven large spots

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Plate II

Fig. 3. T.S. of uterus of mouse (of cancerous strain) containing a large amount of haemosiderin. Fe (K ferrocyanide+HCL) and eosin. Low power. × 74.

Fig. 4. T.S. of uterus of mouse almost immediately after parturition, showing extravasated blood at site of placenta. Ehrlich's haematoxylin and eosin. Low power. × 21.

Fig. 5. Whole uterus of mouse (of cancerous strain) which has had only one litter. Cleared in cedarwood oil. × 3½.

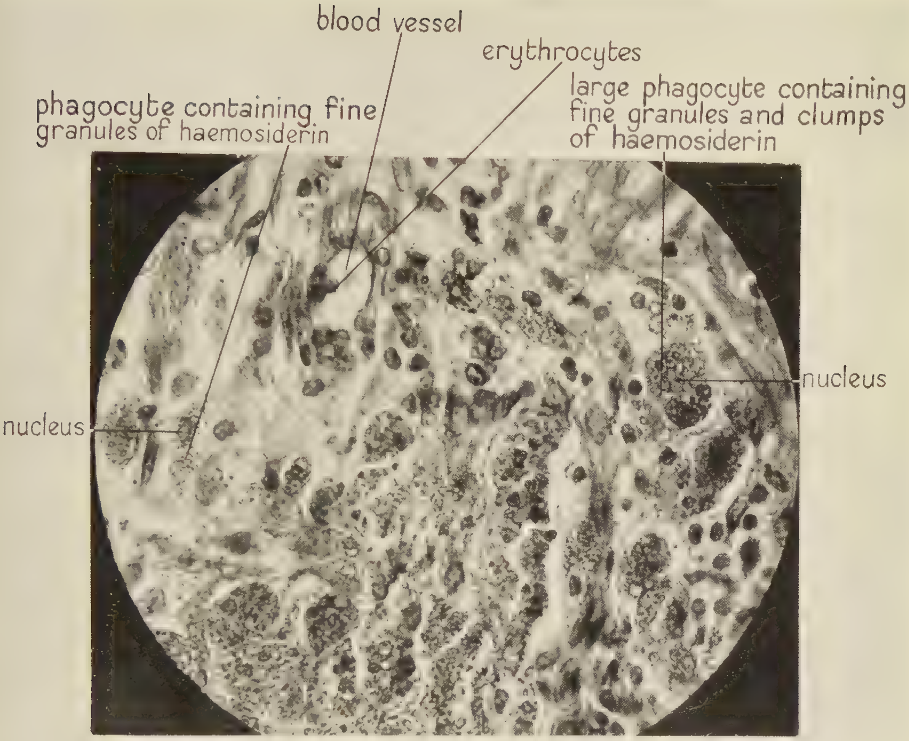
Fig. 6. Whole uterus of mouse (of cancerous strain), 3 days after parturition, which has had two litters. Cleared in cedarwood oil. × 3½.

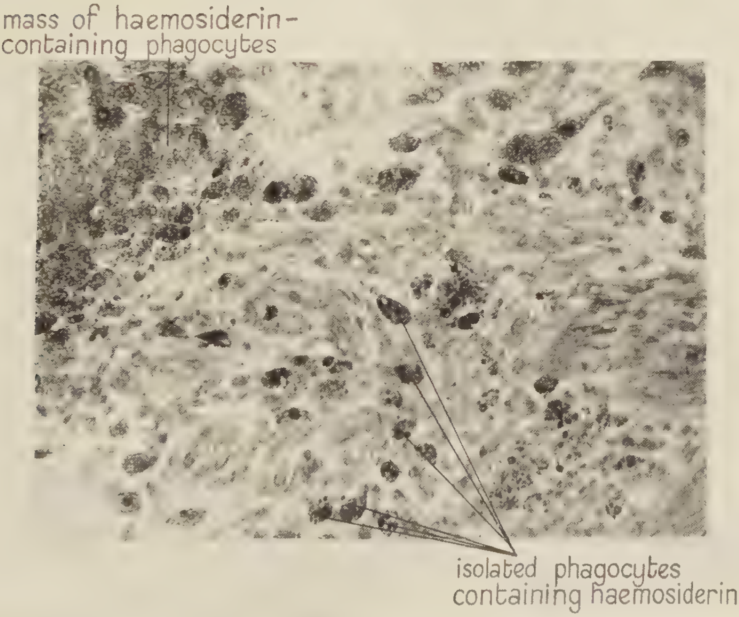
Fig. 7. Whole uterus of mouse of a non-cancerous strain 2 days after its first parturition. Cleared in cedarwood oil. × 3½.

Fig. 8. Whole uterus of mouse of the cancerous strain 2 days after its first parturition. Cleared in cedarwood oil. × 3½.

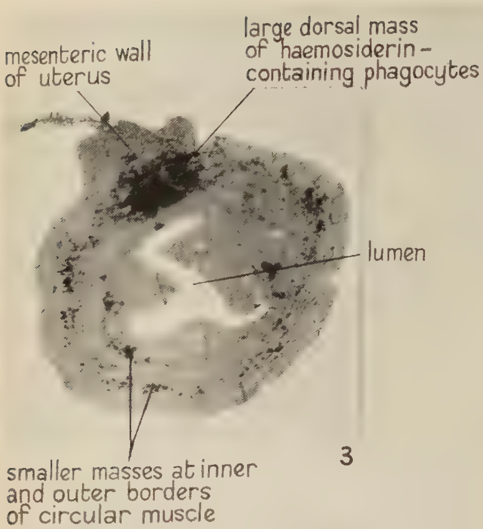
Fig. 9. Whole uterus of mouse of a non-cancerous strain 10 months 7 days after its first parturition. Cleared in cedarwood oil. × 3½.

Fig. 10. Whole uterus of mouse of the cancerous strain over 10 months after its first parturition. Cleared in cedarwood oil. × 3½.

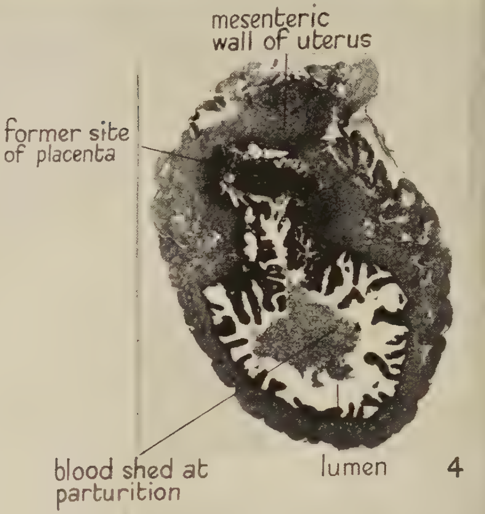

blood vessel
erythrocytes
phagocyte containing fine granules of haemosiderin
large phagocyte containing fine granules and clumps of haemosiderin
nucleus
nucleus


mass of haemosiderin-containing phagocytes
isolated phagocytes containing haemosiderin

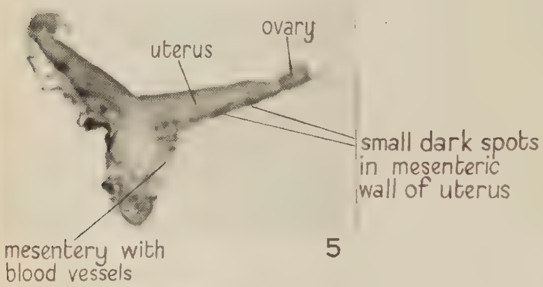
V. JONES.—PLATE 1


mesenteric wall of uterus
large dorsal mass of haemosiderin-containing phagocytes
lumen
smaller masses at inner and outer borders of circular muscle

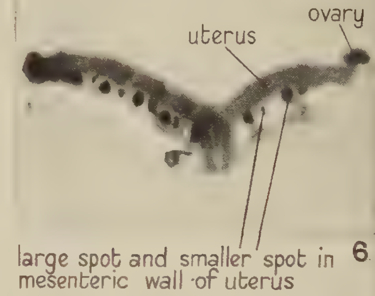
3



4


ovary
uterus
small dark spots in mesenteric wall of uterus
mesentery with blood vessels

5



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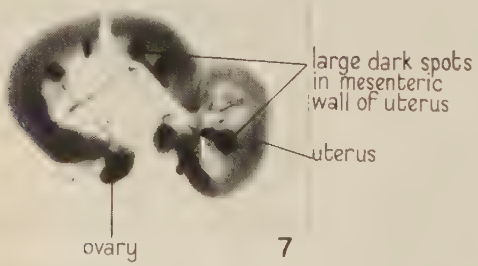

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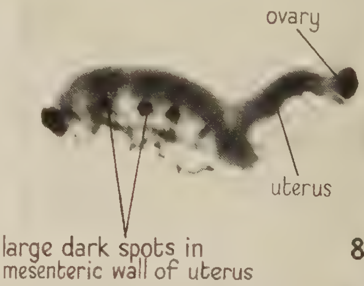

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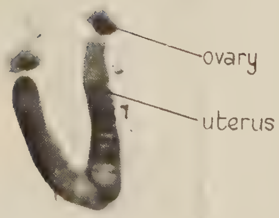
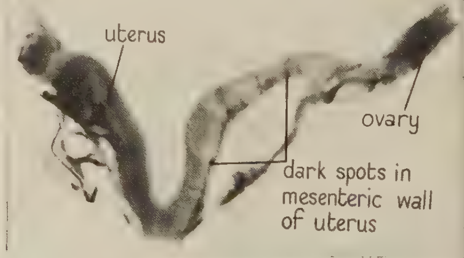


9

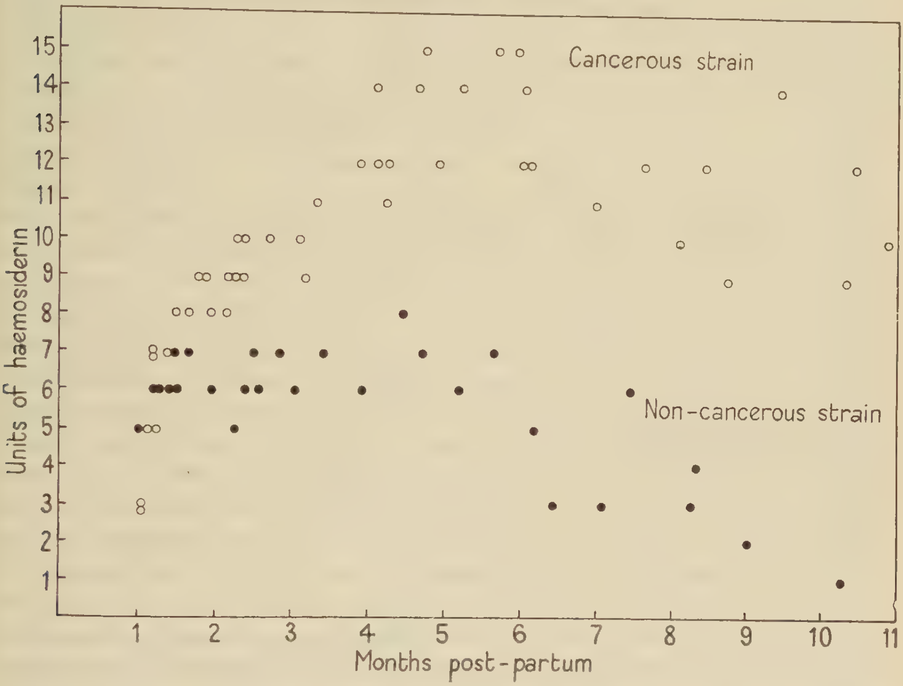


10

V. JONES.—PLATE II

(which represent the placentae of the last pregnancy) are composed chiefly of blood and torn tissue, and the seven small spots (which represent the placentae of the previous pregnancy) consist of haemosiderin.

It seems abundantly clear, therefore, that the dorsal masses of haemosiderin under consideration are formed, after parturition, in the regions of former attachment of the placentae—probably from the blood extravasated into the tissues when the placentae break away at parturition.



TEXT-FIG. 2. Relative amounts of haemosiderin at various periods after parturition in the uteri of cancerous and non-cancerous mice.

Incidence and Relative Amounts of Haemosiderin in Uteri of Mice from Cancerous and Non-cancerous Strains at Various Times after Parturition

Since there was the ever-recurring suggestion that the cancerous strain shows more haemosiderin or shows it more frequently than the non-cancerous strain, a survey was made of the uteri of mice of different strains taken at various times after parturition, from 1 month to 10 months. The results of this survey are shown in Text-fig. 2. No attempt has been made to indicate the exact amounts of haemosiderin present—the estimates are very rough indeed. One unit represents the smallest amount of haemosiderin found in any one uterus, and therefore Text-fig. 2 gives *relative* amounts only. Where the haemosiderin is compact, the area of the largest section of the mass has been measured: where it is less compact, the area of the various parts have been measured. No attempt has been made to calculate the volume, so that the figures given understate the difference in amount. In actually examining the material the differences are very striking indeed.

From the data given in Text-fig. 2 it is evident that almost throughout the series there is much more haemosiderin in the uteri from the cancerous strain than in those from the non-cancerous, taken at corresponding times after parturition. The difference is not apparent, however, till about 1½ months after parturition. Up to that time the uteri of the cancerous strain contain about the same amount of pigment as those of the non-cancerous strains—possibly less. After about 1½ months the amount increases very considerably in the cancerous strain, until about 6 months, but not appreciably in the non-cancerous. After 6 months there is a very definite decrease in amount in the non-cancerous strain but very little decrease in the cancerous strain.

These facts are clearly illustrated in Pl. III, figs. 11 to 15. At 1 month 6 days after parturition (Text-fig. 2) the two uteri (of cancerous and non-cancerous strains) contain approximately the same amount of haemosiderin, although in the non-cancerous strain the pigment is more dispersed, but at 3 months after parturition the cancerous strain uterus (Pl. III, fig. 12) shows a very great amount of haemosiderin as compared with that of a non-cancerous strain (Pl. III, fig. 11). The pigment in the non-cancerous strain uterus is spread over as great an area as that in the cancerous strain, but again is very loosely distributed. Pl. III, fig. 13, shows a uterus of a non-cancerous strain 7 months after parturition and Pl. III, fig. 14, shows one from a cancerous strain at 8 months. Again, the haemosiderin is very much greater in amount in the cancerous strain, and much more compact. By 9 months after parturition the non-cancerous strain uterus shows only a very small amount of haemosiderin (Pl. III, fig. 15) and by 10 months only the merest trace remains—not sufficient to show satisfactorily in a photograph, whereas in the cancerous strain the uteri still show a very large amount of pigment. This decrease in the amount of haemosiderin in the non-cancerous strain and its retention in the cancerous strain is borne out by examination of cleared whole uteri taken at 10 months and more after parturition. In the non-cancerous-strain uterus shown in Pl. II, fig. 9, taken 10 months after parturition, there is no trace of the dark dorsal masses (the small trace found in sections is not visible to the naked eye), whereas in the uterus of the cancerous strain taken after a much longer period (exact date unknown, but at least 10 months) the masses are still clearly visible as dark spots close to the mesentery (Pl. II, fig. 10).

It seems to be clearly established, therefore, that at all times after parturition, apart from about the first month and a half, as long as haemosiderin is present at all, it is present in greater quantities in the cancerous-strain uteri than in the uteri of the non-cancerous strains. Further, it seems quite clear that it disappears almost completely after about 10 months in the non-cancerous strains examined, whereas in the cancerous strain it is scarcely diminished in amount at all at that time.

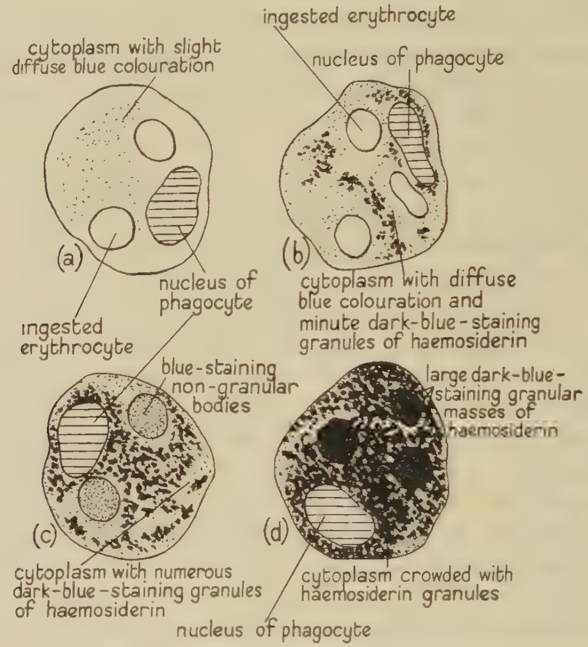
These findings raise several interesting questions. Is less haemosiderin formed in the non-cancerous strains? Or is it dispersed more quickly as it is formed, so preventing the accumulation of large quantities? Is it dispersed at all in the cancerous strain or is it merely more tightly packed as time goes on? In an attempt to throw light on these questions an investigation of the method of formation of haemosiderin was undertaken.

Mode of Formation and Fate of the Pigment in Non-cancerous Strains

For an investigation of the method of formation of haemosiderin within the wall of the uterus, only material from non-cancerous strains was used in the first place, and it is an account of the process as observed in this 'normal' material which is given below.

Immediately after parturition the former site of the placenta shows a considerable quantity of extravasated blood and, a little later, numerous phagocytes can be distinguished ingesting the blood. Cells containing ingested red corpuscles are first observed in uteri taken 12–24 hours after parturition. Ingestion of the erythrocytes seems to be followed within 48 hours after parturition by the appearance of haemosiderin, firstly in a diffuse form and then granular. In material taken at about 48 hours after parturition and stained with hydrochloric acid and potassium ferrocyanide, the phagocytes containing ingested erythrocytes show a diffuse blue coloration of their cytoplasm (Text-fig. 3*a*), and in slightly later material they show minute darker blue granules in the cytoplasm (Text-fig. 3*b*). This suggests that after ingestion some of the haemoglobin diffuses out of the erythrocytes and is broken down into diffuse pigment which is then converted into small granules of haemosiderin. In later material again, stained in the same way, blue-staining non-granular bodies are observed within the phagocytes, similar in shape and size to the erythrocytes (Text-fig. 3*c*) and later still, similar bodies, but of a granular consistency, are to be seen in the same situation (Text-fig. 3*d*) —which seems to indicate that the haemoglobin remaining within the erythrocytes is broken down, *in situ*, into haemosiderin which is at first in a diffuse state and later becomes granular. Phagocytosis of the red corpuscles continues until all extravasated blood is removed from the tissues. This takes a considerable time and cells containing unaltered red corpuscles have been observed as late as 21 days after parturition. Meanwhile, deposition of granular haemosiderin increases day by day until the phagocytes are crowded with the pigment (Pl. I, fig. 1). By 21 days a considerable amount of haemosiderin can be seen in the uterine wall and this continues to increase until about a month and a half after parturition. After this time little or no increase has been observed (see Text-fig. 2, non-cancerous). Increase in quantity of haemosiderin is accompanied by a close aggregation of the pigment-forming phagocytes. These cells, at first widely dispersed, gradually come together into small groups which aggregate to form a loosely packed mass. It should be emphasized that in this non-cancerous material the pigment is never very closely packed (see Pl. III, figs. 11 and 13). No extracellular formation of haemosiderin has been seen. All the pigment has been found within cells except in extravasations of long standing where a small amount of extracellular pigment is present, almost certainly due to disintegration of some of the phagocytes.

It is of interest to compare these observations with those of Muir and Niven (1935). These workers injected fresh mouse blood into mice and studied its fate in the tissues. They observed phagocytosis of red corpuscles within 24 hours after injection and also the appearance of diffuse and granular haemosiderin within the phagocytes from 24 hours onwards. They, too, observed both small granules and large granular masses of haemosiderin and also ingested erythrocytes which gave the Prussian blue reaction. They also


cytoplasm with slight diffuse blue colouration
(a)
nucleus of phagocyte
ingested erythrocyte
ingested erythrocyte
nucleus of phagocyte
(b)
cytoplasm with diffuse blue colouration and minute dark-blue-staining granules of haemosiderin
blue-staining non-granular bodies
(c)
cytoplasm with numerous dark-blue-staining granules of haemosiderin
large dark-blue-staining granular masses of haemosiderin
(d)
cytoplasm crowded with haemosiderin granules
nucleus of phagocyte

TEXT-FIG. 3. Four stages in the formation of haemosiderin within the normal mouse phagocyte. Drawings made from T.S.s of uteri. Fe (K ferrocyanide+HCl) and eosin. Oil immersion. ×2,700.

PLATE III

Fig. 11. T.S. of uterus of mouse of a non-cancerous strain 3 months 28 days after its first parturition. Fe and eosin. Low power. ×40.

Fig. 12. T.S. of uterus of mouse of the cancerous strain 3 months 5 days after its first parturition. Fe and eosin. Low power. ×40.

Fig. 13. T.S. of uterus of mouse of a non-cancerous strain 7 months 12 days after its first parturition. Ehrlich's haematoxylin and eosin. High power. ×275.

Fig. 14. T.S. of uterus of mouse of the cancerous strain 8 months 12 days after its first parturition. Fe and eosin. High power. ×275.

Fig. 15. T.S. of uterus of mouse of a non-cancerous strain 9 months after its first parturition. Fe and eosin. High power. ×275.

state that they observed only intracellular formation of the pigment. Muir and Niven go on to say that at about the seventh day after injection of the blood haematoidin appears in the bodies of the phagocytes and gradually replaces the haemosiderin from which they believe it to be derived. Our own findings, however, show that haemosiderin is the only pigment formed in the mouse uterus from the extravasated blood after parturition: it can still be demonstrated after 10 months in small traces and throughout that period there is no suggestion of haematoidin or of any other pigment anywhere in the uterus. Muir and Niven state that in the period covered by their observations (24 hours to 36 days after injection) they found no haematoidin in phagocytes situated close to blood-vessels or to nerves, haemosiderin being the only pigment found in the cells in these localities, and they add that in the rabbit there was no formation of haematoidin up to 5 days, when their observations ended.

The fate of the haemosiderin in our own material is not known with certainty, but there is a strong suggestion that the phagocytes carry it away from the site of formation. They are never found in the tissues of the uterus surrounding the region of formation, but they are found in the mesentery (Pl. III, fig. 11). It is not certain that they have migrated there from the dorsal mass, but the appearance strongly suggests that they have done so. The dorsal mass seems to 'tail off' into the mesentery and to be continuous with a 'stream' of phagocytes within the mesentery. At the same time the appearance of the dorsal mass changes in a way which suggests bodily removal of some of its constituents. The actual size of the region in which the haemosiderin occurs remains about the same, but the material looks more loosely packed as time goes on until, when only a trace of the pigment remains, it is seen, in section, as tiny clumps of granules scattered over the original area as in Pl. III, fig. 15. Dark blue granules are visible but nothing is clear-cut —they seem to be slightly bordered by a pale blue diffuse stain. On first examining sections in this condition it was thought that the method of preparation had partly dissolved the granules, but this is not the case: every section showing the haemosiderin during its late history shows this blurred appearance. It looks as though there might be a slight diffusion of the pigment immediately around the granules, but the matter is very uncertain. The whole picture of the process, as gleaned from sections, much more strongly suggests bodily removal of the phagocytes with their loads of pigment.

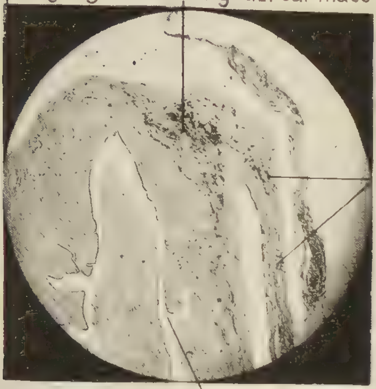
Comparison of the Cancerous and Non-cancerous Strains, with regard to the Mode of Formation and Subsequent History of Haemosiderin in the Uteri

In the present study, after the process of formation of haemosiderin and its subsequent disappearance had been followed in the uteri of non-cancerous strains of mice, attention was turned to the process in the cancerous strain. The basic procedure was found to be the same in both, but certain differences stand out very clearly. In the first place, ingestion of red corpuscles by the phagocytes seems to be slower in the cancerous than in the non-cancerous strains. In the former, it does not begin at the earliest until after 24 hours after parturition—in some not until after 48 hours; whereas in the non-cancerous strains it is clearly marked between 12 and 24 hours. Further, in the cancerous strains phagocytosis can be observed to be still taking place a month or so after parturition, whereas in the non-cancerous strains no trace of it is found after 21 days. The appearance of haemosiderin is also delayed in the cancerous strain until nearly 3 days, sometimes until 4 days, after parturition, whereas in the non-cancerous strains it is unmistakably present at 48 hours. The amount of haemosiderin gradually increases, but again very much more slowly in the cancerous strain. Comparison between a cancerous-strain uterus and a non-cancerous-strain uterus at 21 days after parturition shows very much less haemosiderin in the cancerous strain. But by 1 month 6 days the two strains show approximately the same amount of pigment, although it is more compact in the cancerous strain than in the non-cancerous.

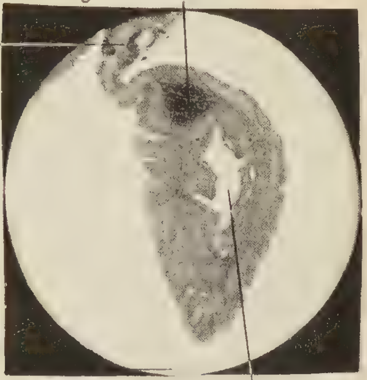
The later history of the pigment in the two strains has already been dealt with above (see p. 483 and Text-fig. 2). In the cancerous strain the amount of haemosiderin continues to increase until 5 or 6 months after parturition and thereafter shows a very slight decrease, but is still not much below its maximum at almost 11 months. In the non-cancerous strains, on the other hand, there is very little increase after the first month, the amount remaining fairly constant until 5 to 6 months, when it begins to decrease, and by 10 months it has practically disappeared. The history of the haemosiderin in the cancerous strain is not known after 11 months. One uterus only has been obtained which was known to be more than 11 months post-partum and unfortunately the exact period is not known, but in the cleared specimen the dorsal masses of haemosiderin were still clearly visible to the naked eye.

Increase in quantity of haemosiderin is accompanied in both strains by a closer aggregation of the pigment-carrying phagocytes, but in the cancerous strain this aggregation is much more marked than in the non-cancerous strains —the packing is very much tighter. Compare Pl. III, figs. 12 and 11, and 14 and 13, showing uteri of cancerous and non-cancerous strains in pairs of comparable ages. The tightly packed masses in the cancerous strain (Pl. III, figs. 12 and 14) contrast strikingly with the loosely aggregated masses in the non-cancerous strains (Pl. III, figs. 11 and 13). This solid packing of the haemosiderin is as characteristic of the cancerous strain as is the excessive amount of the pigment and its prolonged retention.

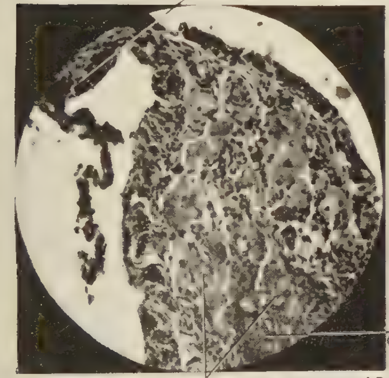
There is not the same suggestion of migration of pigment-carrying phagocytes from the dorsal mass into the mesentery in the cancerous strain as there is in the non-cancerous strain. In the cancerous strain the dorsal mass does not 'tail off' into the mesentery; neither are pigment-carrying phagocytes found within the mesentery (cf. Pl. III, figs. 12 and 11). The occurrence of migration in the non-cancerous strains and its absence in the cancerous strain would account for the closer packing of the haemosiderin in the cancerous strain and for the greater amount, and for its prolonged retention.


scattered clumps of haemosiderin-carrying phagocytes forming dorsal mass
"stream" of pigment carrying phagocytes in mesentery
lumen

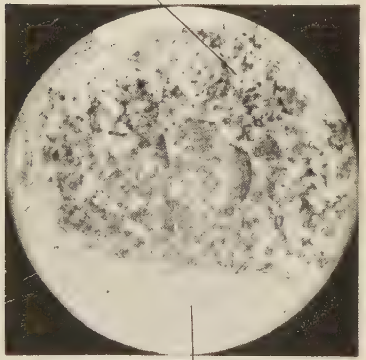
11


large clump of haemosiderin-containing phagocytes forming dorsal mass
mesentery
lumen

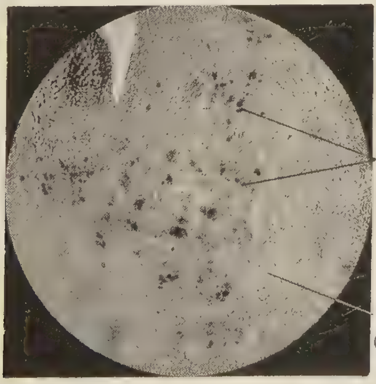
12


mesentery
mesenteric wall of uterus
clumps of haemosiderin-containing phagocytes

13


large mass of haemosiderin-containing phagocytes
mesenteric wall of uterus

14



15

V. JONES.—PLATE III

The whole question of migration requires further investigation and the material available is not what is necessary for the purpose. In most cases the uteri used in this study were dissected out fairly cleanly and only a little mesentery remained attached: no mesenteric lymph glands have been preserved, and the other organs of the body are not available.

CONCLUSIONS AND DISCUSSION

From the foregoing account certain facts emerge clearly. In the first place it is shown that haemosiderin is formed, in all the strains of mice examined, from the blood which is extravasated into the wall of the uterus when the placenta tears away at parturition. In this connexion the most striking facts which emerge, and which are established beyond question, are that in the cancerous strain this haemosiderin is found in much greater quantities than in the non-cancerous strains, and that it is formed somewhat later and persists very much longer. It is by no means certain that these differences are significant with reference to susceptibility to cancer, but they raise some interesting questions and encourage speculation.

In the first place it is not perfectly clear why there is such an excessive amount of haemosiderin in the cancerous strain. Is it that more blood is extravasated into the tissues? Or is it that the breakdown product is disposed of more slowly and so accumulated? The latter alternative seems to be the more probable. There is no evidence whatever of greater haemorrhage at parturition in the cancerous strain, whereas there is abundant evidence of delay in dealing with the extravasated blood. Ingesting of the erythrocytes starts later and goes on for a longer period and the breakdown pigment appears later and persists much longer on the site of formation, as though the phagocytes either fail to carry it away or are extremely slow in the process. It is as though the scavenging is in some way inefficient—as though the phagocytes are either fewer in number or less effective in action. There is no evidence that they are fewer. Histological study shows no scarcity of phagocytes on the scene of action; neither do blood counts suggest any paucity of leucocytes in general, though this latter would, of course, be difficult to demonstrate because of the wide range of variation in normal healthy material. The whole picture, however, suggests slowness or inefficiency of action on the part of the phagocytes rather than paucity in numbers.

An interesting speculation presents itself with regard to susceptibility to cancer. The hypothesis of cellular resistance to cancer put forward by J. B. Murphy (1912, 1913, 1914, 1915, and 1918) and discussed by many later workers, suggests a connexion between the activities of the lymphocytes and susceptibility to cancer. He states that infiltration by lymphocytes accompanies the appearance of a malignant growth. He also says that there is a relationship between the amount of lymphocytic infiltration and the success of a tumour graft—lymphocytosis reducing the susceptibility to tumour transplantation, and lymphocytic leucopenia producing increasing susceptibility. Murphy also found that pieces of spleen introduced into tissue cultures or implanted in a developing hen's egg interfered with the growth of a tumour.

If the phagocytes, or even the reticulo-endothelial system in general, are indeed responsible for resisting the development and growth of cancerous tissue, and if (as the results of the present investigation suggest) this mechanism exhibits inefficiency in the cancerous strain of mice, this might conceivably be the basis of their susceptibility to cancer.

Acknowledgements

I should like to express my gratitude to Mrs. R. C. Bisbee, Department of Zoology, University of Liverpool, for her continued advice and encouragement throughout the course of the investigation.

I wish also to thank Mr. R. A. Fleming of the Zoology Department, University of Liverpool, and Mr. N. Gruber of Reading University National Institute for Research in Dairying, who were responsible for the photography.

Summary

1. In mice, haemosiderin is formed in the wall of the uterus from the blood extravasated into the tissues when the placenta breaks away at parturition.

2. The pigment is formed, probably exclusively, within the phagocytes, and forms a mass in the mesenteric wall of the uterus.

3. In mice of the cancerous strain used in this study (R III strain of Dobrovolskaia-Lavadskaia) the haemosiderin is formed more slowly and is disposed of much more slowly than in the non-cancerous strains examined.

4. Phagocytic inefficiency in the cancerous strain is suggested and its possible connexion with susceptibility to cancer is discussed.

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Some Applications of Phase-contrast Microscopy

BY

ROBERT BARER

(*From the Department of Human Anatomy, Oxford*)

With four Plates and three Text-figures

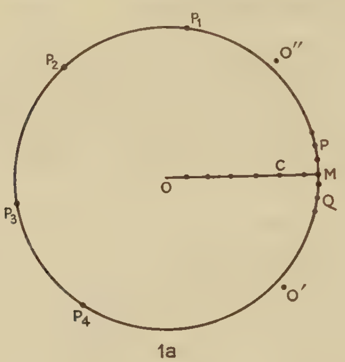
THE purpose of this paper is to present a preliminary report on certain less obvious applications of phase-contrast microscopy which may be of some biological interest. The principles of this method of microscopy are now too well known to necessitate any detailed description (Zernike, 1942; Burch and Stock, 1942).

Briefly, the method sets out to render transparent objects visible by converting changes in phase, which cannot be appreciated by the human eye or by the photographic plate, into changes of amplitude, which can be so appreciated. The theory, as worked out by Zernike (1942), shows that for the case of a grating composed of non-absorbing bars of different refractive indices, the final image is exactly that which would ordinarily be produced by a grating composed of absorbing bars, *provided that the changes in phase produced by the grating are small.* Perhaps the most remarkable feature of Zernike's theory, as applied to a grating, is that it shows the enormous superiority of phase-contrast illumination over other methods for examining transparent material. Without going into details, the image produced by an absorbing grating can be represented by the sum of an infinite number of sine and cosine waves. The image of a transparent grating can be represented by a different series of waves. Ideally we should like to convert this second series into the first, and this is exactly what phase-contrast illumination does! All the other common methods of observation—oblique illumination, central and oblique dark ground illumination, or observation with reduced substage aperture produce images which are represented by different sine and cosine series, which while in some cases showing certain similarities to the 'ideal' series, in general fall far short of it. Roughly speaking, we may say that the effect of phase contrast illumination is as if we stained the object with a dye which stained each point with an intensity proportional to the product of its thickness and refractive index. To this extent, and subject to the performance of the microscope, phase-contrast illumination gives a true representation of what is actually present. All other methods commonly employed to render transparent objects visible produce an image which is often a mere caricature of the object.

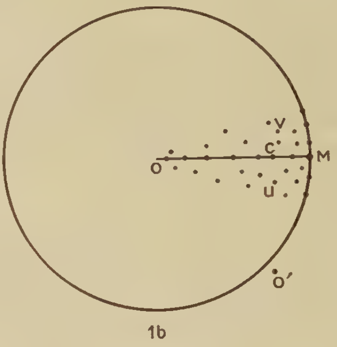
It must be pointed out that this almost exact theory has only been worked out for very special objects, such as gratings, the elements of which produce very small changes of phase. It is reasonable to ask how small must these changes be, and what happens if they become large. It is not easy to give an exact answer to these questions since the mathematics involved is rather complicated. Fortunately, we can tackle the whole question in a much more general way, and without assuming any particular type of object.

Following Zernike's treatment the object (or the image) can be represented by a vector diagram as in Text-fig. 1*a*.

Each point is represented in phase and amptitude by a vector. Areas which absorb light without affecting its phase will be represented by vectors lying along the line *OM*. Areas which absorb no light but merely alter its phase (i.e. transparent objects) will be represented by points on the circumference of a circle. Now according to the Abbe theory of the microscope, the final image is produced by interference between the central undiffracted image of the object and a series of diffraction images. In order to obtain phase contrast it is necessary to change the phase of the central pencil relative to the phases of the diffracted pencils. In practice this is usually accomplished by inserting a phase-retarding or phase-advancing strip into the back focal plane of the objective, and an illuminating diaphragm of corresponding shape in the substage. The phase strip is usually constructed to produce a 90° change of phase in the central image. The reason for this is that the exact theory for the case of a grating requires such a phase change in order to transform a 'phase-grating' into an 'amplitude grating'. In practice, however, and in dealing with a more general type of object, this phase change need not be exactly 90°. Indeed in some cases it is advantageous to produce a phase change other than 90°.



TEXT-FIG. 1*a*.



TEXT-FIG. 1*b*.

Returning to our vector diagram (Text-fig. 1*a*) the effect of the central pencil alone can be represented by a vector which is the average of all the vectors representing the object. Let us suppose it to be represented by the line *OC*. The effect of the phase strip is to rotate this vector through 90°, or in other words to shift the origin of the system from *O* to *O′* (positive phase contrast) or *O″* (negative phase contrast). We can now see that non-absorbing points, which are represented by vectors such as P and Q on the circumference of the circle, will appear to have very different intensities when the origin is shifted to O' or O''. Whereas with normal illumination the intensities of such points were represented by $(OP)^2$ and $(OQ)^2$, i.e. equal and therefore showing no contrast, with phase-contrast illumination the intensities become $(O'P)^2$ and $(O'Q)^2$ or $(O''P)^2$ and $(O''Q)^2$. Restricting ourselves to positive phase contrast (i.e. origin at O') we see that highly refracting points will appear darker, less refracting points lighter, than the mean illumination.

Now suppose the object contains many points which produce large changes in phase. The effect of such points will be represented by vectors OP_1, OP_2, OP_3, OP_4, &c., around the circumference of the circle. If these points are sufficiently numerous the point representing the average of the whole system will approximate to O. Thus phase-contrast illumination would be of no value for such an object. At the same time we observe that the intensity of a point producing a phase change θ will be the same as the intensity for points producing phase changes of $2n\pi+\theta$, i.e. an extremely refractile region may not always appear any darker than a less refractile region.

OBSERVATIONS ON LIVING INTACT ANIMALS

In practice, according to Zernike (1942), phase-contrast illumination is most useful for objects which produce phase changes of less than 45° (one-eighth of a wave-length) since objects thicker than this can be seen reasonably well by other methods. In most cases this means that phase contrast is best used with very thin objects. Single cells such as protozoa or tissue cultures are ideally suited for this method of examination. The excellent films produced by Michel (on the development of grasshopper spermatocytes) and by Hughes (on the growth of cells in tissue cultures) have already shown the enormous potentialities of the method for this type of material. Unfortunately, the insistence on the use of *thin* objects has rather tended to obscure the point that it is not thickness *per se* that is important, but the amount of phase change. Phase-contrast illumination may be quite useful for examining relatively thick but very transparent objects. Now the larva of *Chaoborus* (the phantom larva) has long been known for its transparency, and it was thought that it would provide good material for examination by phase-contrast illumination. The larva is usually several millimetres in diameter but can be flattened somewhat by mounting in water under a cover slip. At the same time this immobilizes the insect, but if carried out gently does not appear to harm it, for the latter will swim away quite normally when released after examination. Now it must be admitted that many of the cells and tissues of *Chaoborus* can be observed by the familiar method of reducing the substage iris to a very small diameter and altering the focus. As a matter of fact this is one method (though a very poor one) of producing phase contrast. Resolving power is lost owing to the small substage aperture, the method only works properly when the object is slightly out of focus, diffraction effects are accentuated, and the image is a very poor representation of what is actually present. With properly adjusted phase-contrast illumination the results are very different. It is possible to state with reasonable confidence that we can now observe living cells in the living intact multicellular organism with greater clarity, and with a greater chance of seeing what is actually present, than ever before. Pl. I, fig. 1, shows a low-power general view of part of a living *Chaoborus* larva, focused on two ganglia with the intervening nerve-cord. Pl. I, figs. 2 and 3, are higher power views of single ganglia, taken with the $\frac{1}{6}$ in. objective. These photographs show one large nerve-cell on each side of the ganglion. The nuclei and nucleoli are clearly seen and it will be observed that the cytoplasm is highly granular. These granules are particularly well seen in Pl. I, fig. 3. It is not yet possible to establish their nature with certainty. At first sight they might be taken for Nissl granules, and indeed their appearance in Pl. I, figs. 2 and 3, closely resembles that in fixed sections stained with Borrel's methylene blue, a recognized Nissl stain. It is possible, however, that the granules seen in the living cells may be part of the Golgi apparatus, which is often very easily seen in other living cells by phase-contrast illumination. Another region of some interest is shown in Pl. I, fig. 4. It is necessary to speak with caution of such material as the tissues of living insect larvae, but as a result of prolonged observations I am of the opinion that Pl. I, fig. 4, is a photograph of a living motor nerve-ending in a muscle-fibre. (All the muscles of *Chaoborus* larvae are composed of single fibres.) The pyramidal accumulation of sarcoplasm containing one or more nuclei with what appears to be a nerve-fibre entering at the apex corresponds to the description of the 'Doyères hillock' given in the entomological literature (see Morison, 1927). Pl. II, fig. 5, shows another view of these end-plates taken at the same magnification but from a smaller specimen. Two end-plates are seen (they lay on slightly different planes and a compromise focus had to be chosen in order to show both on the same photograph) and they appear to be interconnected by two nerve-fibres which seem to emerge from a common junction, at which there appears to be some sort of cell, possibly a peripheral nerve-cell, but more probably a neurilemmal cell. Numerous sarcoplasmic granules are also seen. Other insect larvae are also suitable for study by this method, e.g. young mosquito and *Chironomus* larvae (Pl. II, fig. 6). The giant salivary gland chromosomes in the intact *Chironomus* larva can often be seen quite well. Numerous possibilities exist for the study of secretory and excretory activity of cells in insect larvae. It is very much to be hoped that suitable vertebrate material may be found. I have carried out preliminary observations on the tissues of a tadpole's tail and the results were quite promising. Unfortunately, these observations were made rather late in the spring when frog tadpoles were very scarce and rather large. The transparent tails of very young tadpoles should be quite suitable. The mesenteries of small vertebrates have not on the whole been found satisfactory, though very clear views of the capillary endothelium have sometimes been obtained. It is possible that a very thin transparent chamber inserted in the ear of a rabbit may prove satisfactory.

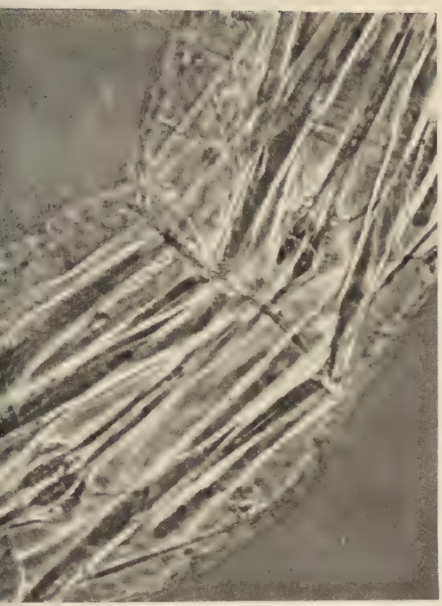


FIG. 1

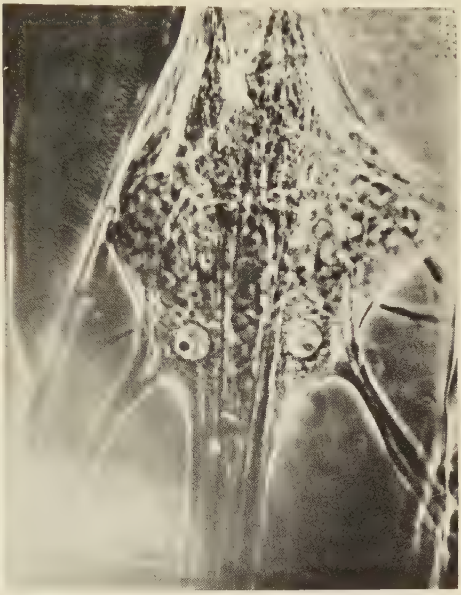


FIG. 2

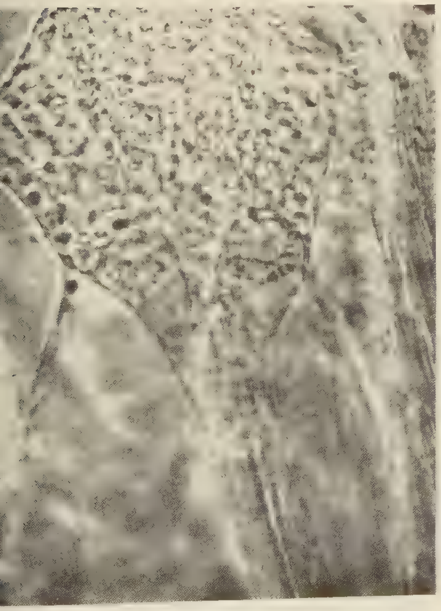


FIG. 3

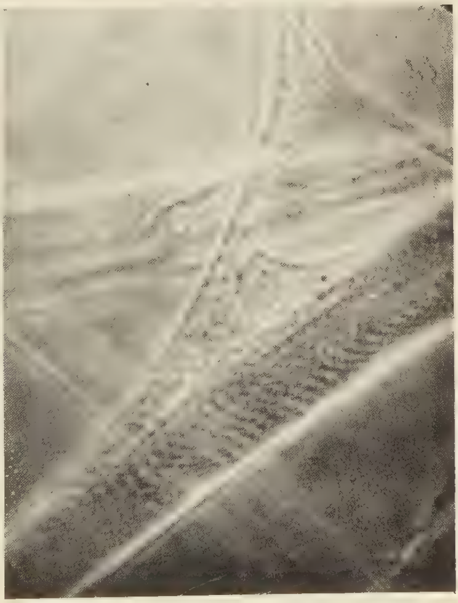


FIG. 4

R. BARER.—PLATE I

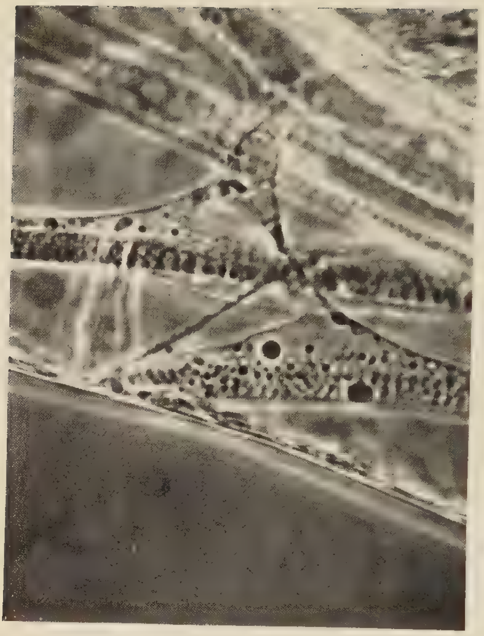


FIG. 5

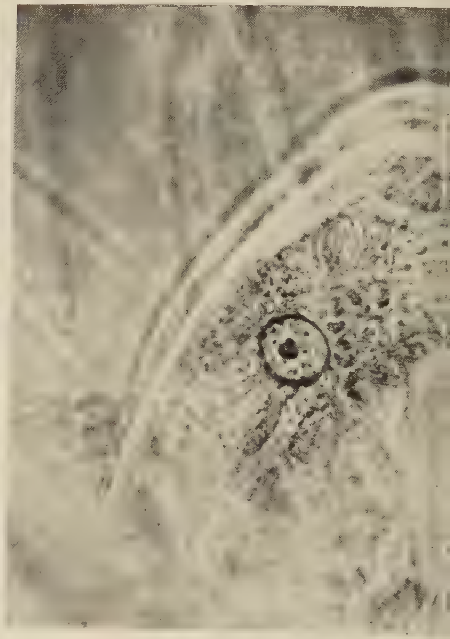


FIG. 6

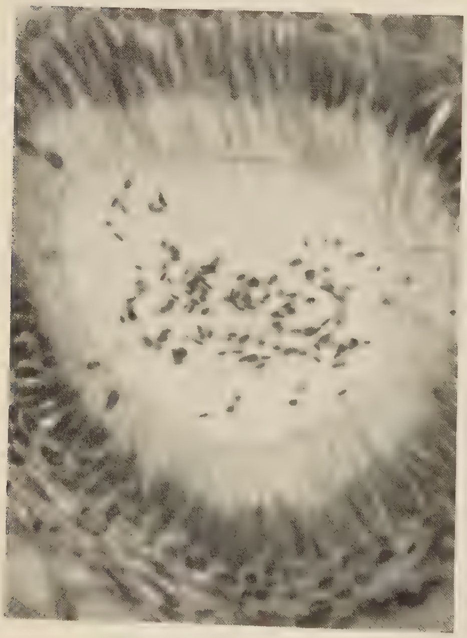


FIG. 7

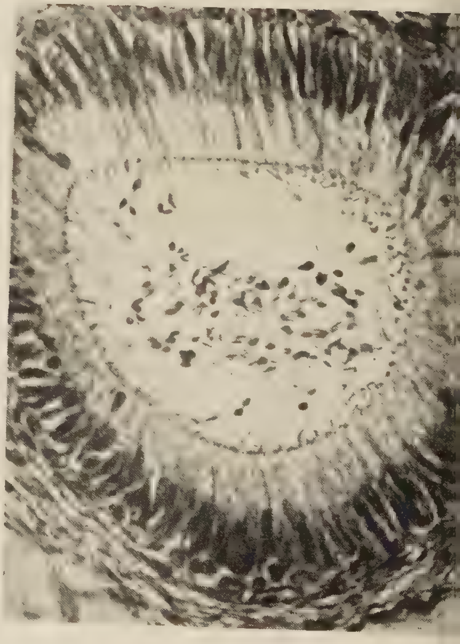


FIG. 8

R. BARER.—PLATE II

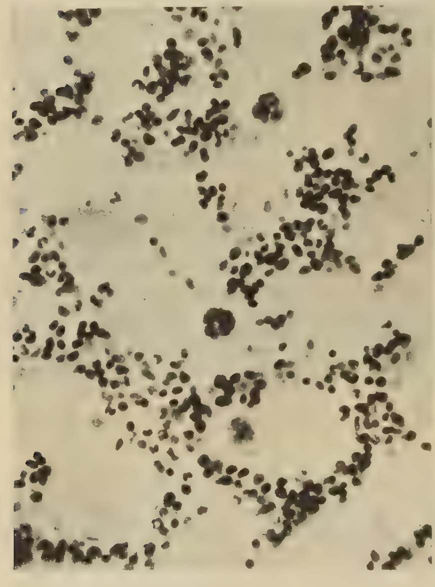
THE EXAMINATION OF STAINED SECTIONS

It is generally stated that phase-contrast illumination is of no value for examining stained objects. This opinion seems to have been based mainly on theoretical consideration of an over-simplified ideal case. In Text-fig. 1 we saw that Zernike represented all absorbing points as lying on the line OM, and all phase-changing points as lying on the circumference of the circle. When the origin is shifted from O to O', the absorbing points on OM become roughly equidistant from O' and therefore lose contrast. Now a stained section will contain points like U and V (Text-fig. 1*b*) which both absorb and produce changes of phase. The intensities of such points will be proportioned to $(O'U)^2$ and $(O'V)^2$ and they will appear to have good contrast, whereas with ordinary illumination (origin at O) they will appear equally stained. We thus have the theoretical possibility that phase-contrast illumination may after all be of some value for examining stained objects. To put the matter very crudely we should expect to lose much of the contrast due to the staining, but we might gain a different contrast due to differences in refractive index in both the object and the stains. (In a private communication Dr. Burch has suggested that stains may have anomalous refractive indices in the region of their absorption bands, and that it would be interesting to examine stained sections by phase-contrast illumination using monochromatic light of different wave-lengths, in order to vary staining contrast.) It must be admitted at once that this simple theory is far from adequate. Unfortunately, the exact treatment bristles with difficulties, and, indeed, it is unlikely that the problem will ever be solved for any but very special cases. In the circumstances it was judged best to try the method in practice. A wide range of routine normal and pathological material was examined, but a systematic examination of different stains and mounting media has not yet been made.

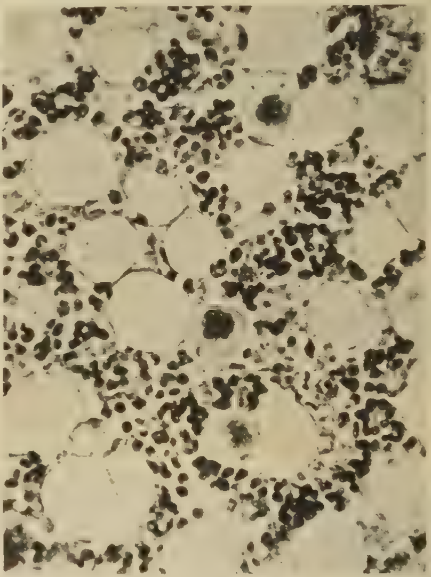
There are several ways in which phase-contrast illumination may be of value for examining stained sections. Perhaps its most obvious use is to render visible details of structure which have not been properly stained by the dyes employed. Such cases occur especially in certain histochemical reactions. Text-fig. 2*a* shows a section of bone marrow stained by the Feulgen technique. Only the nuclei are visible. Text-fig. 2*b* shows the identical field as seen by phase-contrast illumination. The cytoplasm as well as the nuclei of the cells is now rendered visible.

Other examples of the way in which unstained or poorly stained details can be made clearer are shown in Pls. II and III, figs. 7–10. It should be pointed out that it is often extremely difficult to demonstrate the superiority of phase-contrast illumination over ordinary illumination by means of photographs. The reason for this is that one automatically tries to get the best possible photograph out of any given material. Details which are seen with difficulty through the microscope can be rendered clearer by filters, differences in exposure, development, and printing. Strictly speaking one ought to treat both phase-contrast and ordinary pictures identically, but this is very difficult as the former require longer exposures. A direct comparison under the microscope is far more convincing than any series of photographs. Were it not for a natural tendency to 'load the dice' against oneself the photographic superiority of phase-contrast would be even more clear-cut.

Neurological material offers a particularly interesting field for study by means of phase-contrast microscopy. It is well known that scarcely any two methods of staining nervous tissues will produce the same result. As regards the various methods of silver staining, one may almost say that the same method in the hands of two different workers will produce different results. It is, therefore, of some interest to know what an entirely unstained nerve-cell looks like. The answer is seen in Pl. III, figs. 11, 12.



TEXT-FIG. 2*a*.



TEXT-FIG. 2*b*.

These cells were almost invisible by ordinary illumination, even when the substage iris was almost closed, and were quite impossible to photograph, except by phase-contrast illumination. The appearance is more or less what one might expect to see if it were possible to use a mixture of silver staining and Nissl staining. It is the purpose of this paper to describe the possible applications of a new technique rather than to discuss results, but for the present we may say that the nerve-cell, free from staining artifact (but not, of course, free from fixation artifact), may show appearances suggestive of Nissl granules, Golgi apparatus, *boutons terminaux*, and numerous extremely fine cell processes. A further investigation on frozen-dried material and tissue cultures is being undertaken.

Returning, however, to the question of stained nervous tissue, in general

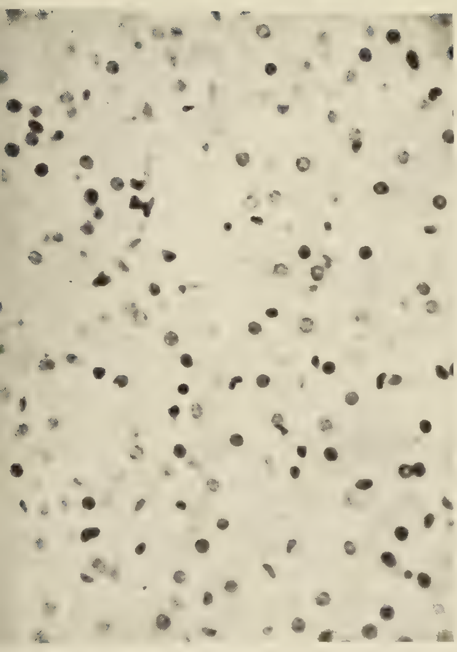


FIG. 9

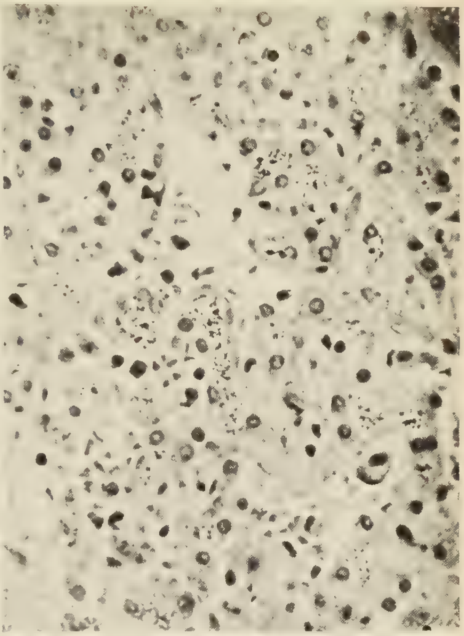


FIG. 10

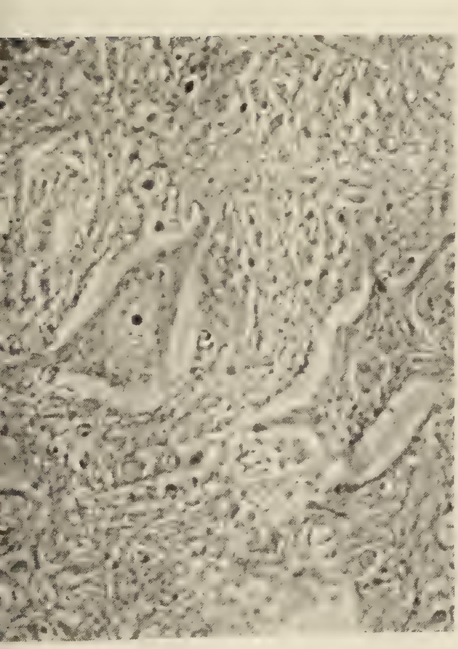


FIG. 11

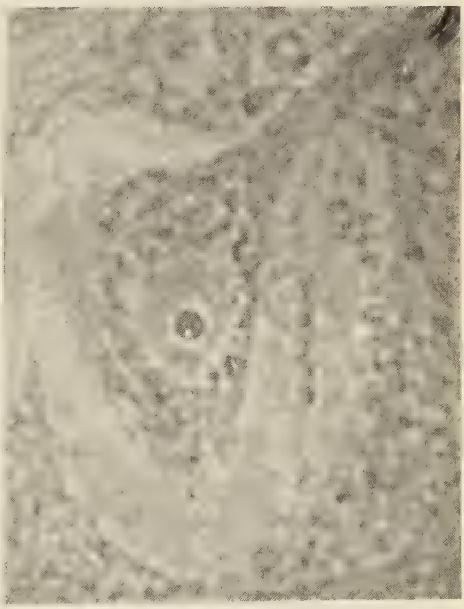


FIG. 12

R. BARER.—PLATE III

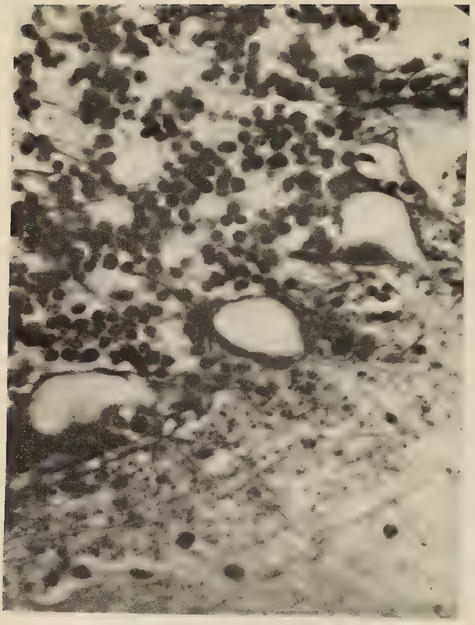


Fig. 13

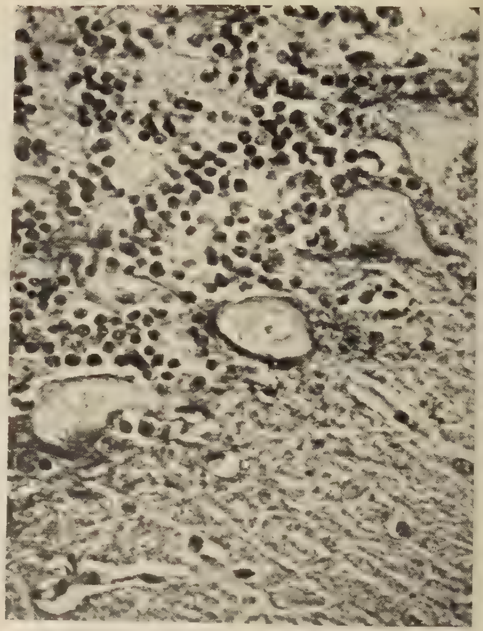


Fig. 14

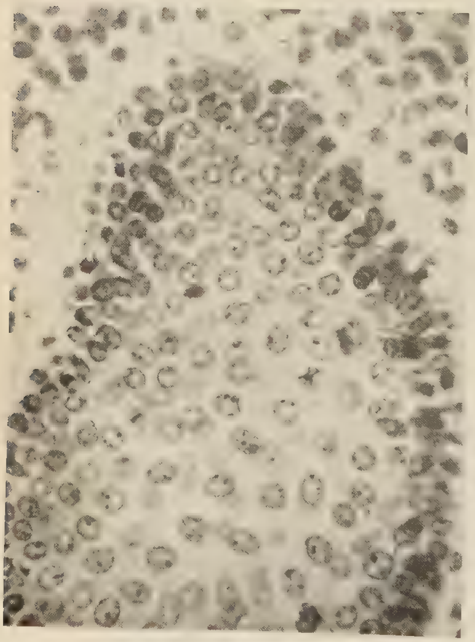


Fig. 15

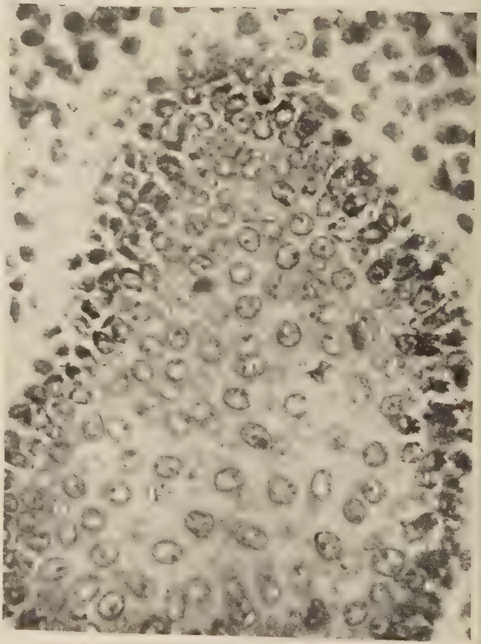
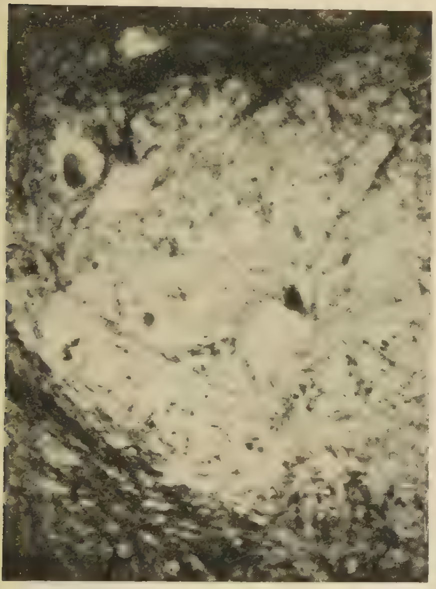


Fig. 16

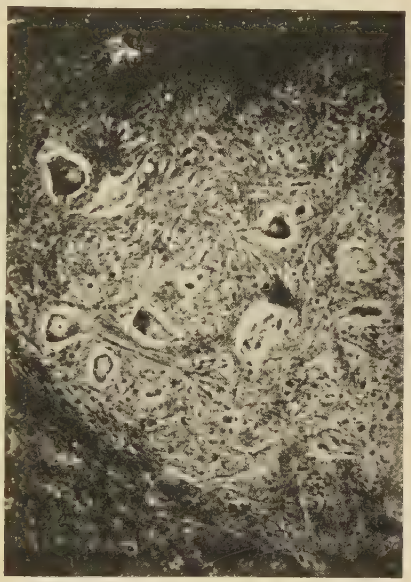
R. BARER.—PLATE IV

we may say that any given stain is fairly specific for either nerve-cells or nerve processes. Thus toluidin blue, methylene blue, and cresyl violet stain nuclei and Nissl granules but not nerve-fibres, whereas the reverse is on the whole true of most silver stains. We thus have the possibility of rendering the missing element visible by phase-contrast illumination.

Pl. IV, fig. 13, shows the Purkinje cells of the cerebellum seen in a Bielschowsky preparation. The nerve-fibres are very clear but the cells seem almost devoid of detail. Pl. IV, fig. 14, shows the same field by phase-contrast illumination. The nuclear structure is clearly visible. Conversely it is often possible to render the nerve-fibres more visible in Nissl preparations by means of phase-contrast. Finally, it is necessary to mention the various myelin stains, used for studying nerve-tracts. These usually leave the nerve-cells unstained and the latter can be rendered visible by phase-contrast illumination. Text-figs. 3*a*, 3*b* show this effect in a Weigert-Pal preparation (× 150) of a transverse section of the spinal cord (monkey).



TEXT-FIG. 3*a*.



TEXT-FIG. 3*b*.

The main disadvantage of the method is that it very often renders too much detail visible. Most stains are to a certain extent selective, but phase-contrast illumination may reveal so many minute fibrils that it is very easy to miss the wood for the trees. A considerable amount of further study will be necessary before any exact evaluation of the method as applied to neurological material can be made.

Many types of granules are extremely easily seen by means of phase-contrast illumination. Pl. III, figs. 9, 10, shows a section of liver from a case of pernicious anaemia. Iron-containing pigment granules (haemosiderin) are present but very difficult to see by ordinary methods. Phase contrast reveals the presence of myriads of these granules with remarkable clarity. Even such normally well-seen pigment granules as melanin are rendered clearer by phase contrast. Pl. IV, figs. 15, 16, from a section of a melanoma of the skin illustrate this point. The method has also been used with success for demonstrating secretory granules. It should be mentioned that all the photographs of fixed material shown were from routine paraffin sections, 8–12 μ thick, mounted in Canada balsam.

DISCUSSION AND CONCLUSIONS

It is necessary to emphasize once more that it is not the purpose of this paper to discuss results in detail, but rather to suggest certain applications of phase-contrast microscopy which require further study. As regards the examination of living cells and tissues anyone who has ever seen such material by properly adjusted phase-contrast illumination would readily acknowledge its superiority over the older methods. The main problem here is to find suitable material. Isolated cells such as bacteria, protozoa, tissue cultures, or spermatozoa are of course ideal, but multicellular organisms are in many ways more interesting and important. Insect larvae and other transparent creatures such as certain medusae may yield useful information, but it would be very valuable to be able to extend the range of available material, especially to vertebrate tissues. The examination of stained sections by phase-contrast illumination is still in its infancy. Time and further investigation alone will show its uses and limitations. All that can be said at present is that it may prove to be a useful adjunct to the usual methods for examination of certain types of material. It is not possible to predict what will be seen by its aid. In some cases certain details may be rendered more clearly visible, in others the method will be found of no value. One practical point should be emphasized: the appearance of a stained section under the low power ($\frac{2}{3}$ in. objective) is often bizarre and disappointing. The investigator should not be disturbed by this but should always proceed to examine the section with the $\frac{1}{6}$ in. objective, when the appearance is often greatly improved. I am at present unable to offer any satisfactory explanation for this phenomenon, but recent experiments suggest that appearances can be greatly improved by proper choice of colour filters. One last word: it is of course very important that any new method of microscopy should be received with caution. It must be admitted that there are many theoretical and practical points about phase-contrast illumination that require much further study. Nevertheless the theoretical basis underlying the method is sufficiently well understood to enable one to say that the image seen by its use bears at least as close a relation to what is actually present as that seen by more orthodox methods. Indeed, it is necessary to point out that the theory underlying the observation of stained objects by ordinary illumination is very far from complete. The theory underlying the use of oblique or central dark ground illumination is even less perfectly understood, but this has not prevented the extensive use of these methods. The proper course to pursue for the present is to use the method cautiously as an adjunct to other routine methods and to seek out its special applications and limitations.

I wish to thank Professor W. E. Le Gros Clark, F.R.S., for his encouragement and advice. I have had several valuable discussions on histological matters with Drs. A. Brodal, P. Glees, and G. Bourne. Mr. A. W. Dent and Mr. L. G. Cooper have provided technical assistance. Finally, I wish to express my special gratitude to Dr. C. R. Burch, F.R.S., for many stimulating discussions on all aspects of phase-contrast microscopy.

SUMMARY

Some extensions of the simple theory of phase-contrast microscopy are considered. It is emphasized that *transparency*, rather than *thickness*, is the limiting factor for the successful employment of the method. Certain transparent insect larvae (*Chaoborus*, *Chironomus*) can be observed in the living state by phase-contrast illumination.

The statement that the method is of no value for the examination of fixed and stained sections is based on consideration of an ideal physical case. In practice the method may be a valuable adjunct to routine examination of such material. Examples are given of the application of phase-contrast microscopy to normal and pathological stained sections.

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Morison, G. D., 1927. Quart. J. micr. Sci., **71**, 395.
Zernike, F., 1942. Physica, **9**, 686, 974.

EXPLANATION OF PLATES

All the figures are untouched photomicrographs taken with the Cooke, Troughton, and Simms phase-contrast equipment. Green filter. Orthochromatic film.

Plate I

Fig. 1. Low power view (phase contrast × 150) of two segments of a living *Chaoborus* larva. Focused at the level of the nerve-cord and ganglia. Two large nerve-cells are visible in the lower ganglion.

Fig. 2. Higher power view (× 500) of a single ganglion showing two nerve-cell nuclei and nucleoli.

Fig. 3. Another example (× 500) showing the granules in the cytoplasm of the large nerve-cell on the right.

Fig. 4. Motor nerve-ending (phase contrast × 500) in striated muscle-fibre of a *Chaoborus* larva. Note the nucleus.

Plate II

Fig. 5. Another example of motor nerve-endings; same magnification as Fig. 4 (× 500) but from a smaller specimen. The two muscle-fibres lay on slightly different levels and a compromise focus was chosen. Note the sarcoplasmic granules and the apparent inter-connexion of the nerve-fibres.

Fig. 6. Nucleus and nucleolus of a cell in an anal appendage of a living *Chironomus* larva. Phase contrast × 500.

Fig. 7. Epididymis tubule. Haematoxylin and eosin. Normal illumination. × 500.

Fig. 8. Same field. Phase contrast. The details of the poorly stained ciliated epithelium are now clearly seen.

PLATE III

Fig. 9. Liver in pernicious anaemia. Haematoxylin and eosin. Normal illumination. × 500.

Fig. 10. Same field. Phase contrast. The tiny haemosiderin granules are made visible.

Fig. 11. Anterior horn cells of spinal cord of a monkey. Unstained. Alcohol-acetic acid fixation. Phase-contrast. × 500.

Fig. 12. Anterior horn cell of monkey. Unstained alcohol-acetic acid fixation. Phase contrast. × 1,200.

PLATE IV

Fig. 13. Purkinje cells, cerebellum. Bielschowsky's silver impregnation. Normal illumination. × 500.

Fig. 14. Same field. Phase contrast. Note nuclear detail and great increase in number of fibrils.

Fig. 15. Melanoma of skin. Haematoxylin and eosin. Normal illumination. × 500.

Fig. 16. Same field. Phase contrast. Melanin granules become more sharply defined. Note also the intercellular bridges.

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